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VOLUME 37 JANUARY 1959 NUMBER 1

THE ANTHOCYANINS OF SOME CANADIAN PLANTS[1]

R. M. ACHESON[2]

Abstract

The anthocyanins of a number of Canadian flowers and berries have been examined and the anthocyanidins identified. No new types were detected.

Introduction

A number of surveys (1, 5, 6, 7, 9, 10) of the anthocyanins of plants in different parts of the world have been carried out but none have hitherto used material exclusively from Canadian sources. The present investigation concerned an examination of the anthocyanins of some Canadian plants in the hope of finding both new pigments and chemotaxonomic information of the type which has been obtained in investigations of *Papaver* species (2).

Methods and Materials

The plant material was collected in British Columbia and Alberta (see Table I) in the summer of 1957. The petals and berries were placed in tubes containing 1% aqueous hydrochloric acid at the time of collection and were stored at room temperature until brought to Oxford for examination. The aqueous extract was concentrated *in vacuo* at room temperature, when necessary, and chromatographed (ascending solvent) on Whatman No. 1 paper using the organic phase of a *n*-butanol:acetic acid:water (4:1:5 by volume) mixture (2, 4). The homogeneous 4:1:1 mixture, being equivalent for many other compounds (3), was tried with a number of the extracts but caused bad streaking. After drying, the papers were dipped or sprayed with 0.1–0.05% aqueous ferric chloride when spots due to anthocyanins with free ortho hydroxyl groups became clear blue. Care is necessary in differentiating this color from red-violets which are only due to pseudo-base formation. A small number of anthocyanins gave an initial red-violet color which changed to blue on standing. This may be due to the elimination of

[1]Manuscript received July 16, 1958.
Contribution from the Department of Biochemistry, University of Oxford, South Parks Road, Oxford, England.
[2]Department of Biochemistry, Oxford University, England.

a glycosidic residue under the influence of the reagent. The anthocyanin solutions were hydrolyzed by adding an equal volume of concentrated hydrochloric acid and boiling for a few minutes. The aglucones were collected with amyl alcohol and chromatographed (ascending) in the Forestal (4) solvent (concentrated hydrochloric acid:acetic acid:water, 3:30:10 by volume) side by side with synthetic cyanidin and pelargonidin. They were identified by their color reactions and by a comparison of their R_f values with those of synthetic pelargonidin or cyanidin, on the same chromatographic paper, or with published R_f values (1, 4) for other anthocyanidins.

Results and Discussion

The results are given in the table where the plants have been arranged in the order and numbering of Hutchinson (8). In a number of instances two and three anthocyanins derived from the same anthocyanidin are present in the same plant. The results obtained here confirm those recorded earlier (9) for *Chamaenerion* (*Epilobium*) *angustifolium* L. The species *Gentianella* examined contained delphinidin glycosides, as do all members of genus *Gentiana* so far reported (1, 5, 6, 9).

TABLE I

Chromatographic properties of the petal anthocyanins and the aglucones obtained on hydrolysis

Plant	Source	Aglucone identified	R_f values × 100: Of anthocyanidin in solvent A: From anthocyanin hydrolysis	Of anthocyanidin in solvent A: Standard	Of anthocyanin in solvent B
4 Ranales					
15 Ranunculaceae					
Aquilegia formosa Fischer	2	Pelargonidin	75	74	45
14 Saxifragales					
47 Saxifragaceae					
Leptarrhena pyrolifolia (D. Don) R. Br. var. *subalpinus* (Robinson) C. P. Smith	2	Cyanidin	48	49	20,* 34*
21 Lythrales					
77 Onagraceae					
Epilobium latifolium L.	2	Cyanidin	55	54	30
Epilobium adenocaulon Hausskn.	1	Cyanidin	47	47	23,* 34
Chamaenerion angustifolium (L.) Scop.	1	Malvidin	62	60†	32
40 Rosales					
143 Rosaceae					
§*Rubus vitifolius* Cham. and Sch. (=*R. macropetalus* Dougl. ex. Hook.)	1	Cyanidin	49	49	33*
§*Rubus parviflorus* Nutt.	1	Pelargonidin	72	72	41
§*Rubus spectabilis* Pursh red var.	1	Cyanidin	47	49	34*
§*Rubus spectabilis* Pursh yellow var. No anthocyanin could be detected					
§*Prunus emarginata* (Dougl.) Walp.	1	Cyanidin	49	50	20, 26
		Pelargonidin	71	71	33
Spiraea douglasii Hook.	1	Cyanidin	47	49	17,* 27*
148 Papilionaceae					
Lupinus arcticus S. Wats.	2	Delphinidin	32	30†	27*
		Cyanidin	50	50	
	3	Delphinidin	33	30†	26*
		Cyanidin	52	54	
Lupinus lyallii A. Gray	7	Delphinidin	30	30†	25,* 33*,‡

TABLE I—*Concluded*

Plant	Source	Aglucone identified	R_f values × 100: Of anthocyanidin in solvent A — From anthocyanin hydrolysis	Of anthocyanidin in solvent A — Standard	Of anthocyanin in solvent B
60 Ericales					
215 Ericaceae					
§*Gaultheria shallon* Pursh	1	Cyanidin	48	48	23,* 27,* 33*
		Delphinidin	29	30†	
Phyllodoce empetriformis (Smith) D. Don	2	Cyanidin	53	53	31
Pyrola asarifolia var. *incarnata* (DC.) Fernald	2	Cyanidin	49	50	28
§*Vaccinium parvifolium* Smith.	1	Cyanidin	47	49	22
66 Rubiales					
233 Caprifoliaceae					
Lonicera involucrata (Richards.) Banks	4	Cyanidin	50	52	31*
Linnaea borealis L.	5	Cyanidin	50	49	22*
Sambucus callicarpa Greene	1	Cyanidin	53	53	23,* 34*
67 Asterales					
238 Compositae					
Agoseris carnea Rydb.	2	Cyanidin	53	54	35*
Erigeron macounii Greene (= *E. scribneri* Canby)	6	Cyanidin	45	47	07,* 15*
Erigeron callienthemus (=*E. saluginosus* (Richards.) A. Gray)	4	Cyanidin	48	49	09,* 19*
68 Gentianales					
239 Gentianaceae					
Gentianella amarella s. sp. *acuta* (Michx.) J. M. Gillett	10	Delphinidin	31	30†	20,* 30‡
Gentiana glauca (Pallas) Rydb.	9	Delphinidin	31	30†	21,*‡ 29*
71 Campanales					
243 Campanulaceae					
Campanula petiolata DC.	8	Delphinidin	30	30†	12*
72 Polemoniales					
247 Polemoniaceae					
Polemonium columbianum Rydb. (probably)	7	Delphinidin	34	30†	16*
73 Boraginales					
249 Boraginaceae					
Myosotis virginica (L.) B.S.P.	4	Cyanidin	47	47	05*
Mertensia ciliata (Torr.) D.Don	10	Delphinidin	32	30†	10*
		Cyanidin	51‡	50	
75 Personales					
252 Scrophulariaceae					
Castilleja miniata Dougl.	3	Pelargonidin	74	74	37
Pedicularis bracteosa Benth.	9	Cyanidin	53	51	34
	7	Cyanidin	54	54	33
Pedicularis groenlandica Retz.	10	Cyanidin	50	49	29
Penstemon procerus Dougl.	7	Cyanidin	54	54	11,* 21*
Veronica wormskjoldii Roem. and Sch.	9	Delphinidin	30	30†	42*
89 Liliales					
293 Liliaceae					
Lilium montanum A. Nels.	10	Cyanidin	53	53	29
93 Amaryllidales					
306 Amaryllidaceae					
Allium cernuum Roth	11	Cyanidin	54	53	18

NOTE: Sources: 1, vicinity of Vancouver, B.C.; 2, Garibaldi Park, B.C.; 3, Noaxe Lake, B.C.; 4, Yoho valley, Alta.; 5, Waterfowl Lake, Alta.; 6, Peyto Lake, Alta.; 7, Elisabeth Mine, B.C.; 8, Olympic peninsula, Wash.; 9, Tonquin valley, Alta.; 10, Miette Hot Springs, Alta.; 11, Maligne Canyon, Alta.

*Definite pure blue color obtained with 0.05% aqueous ferric chloride indicating free ortho hydroxyl groups in the glycoside.

†R_f quoted from reference 4.

‡Minor constituent barely detectable.

§Anthocyanin obtained from the berry.

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Grateful thanks are due to Dr. V. C. Brink of the University of British Columbia, who very kindly identified all of the specimens and collected some of them, to Dr. A. C. Neish of the Prairie Regional Laboratory, Saskatoon, for gifts of synthetic cyanidin and pelargonidin, and to Dr. J. L. Harper of the Department of Agriculture, University of Oxford, for help in the preparation of this manuscript.

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OXYGEN CONSUMPTION AND BLOOD FLOW IN THE SUBMAXILLARY GLAND OF THE DOG[1]

K. GODWIN TERROUX, P. SEKELJ, AND A. S. V. BURGEN

Abstract

The blood flow and oxygen consumption of the submaxillary glands of seven dogs were measured while the glands were at rest and during secretory activity. Serial samples of blood were delivered directly from blood vessels to the cell of a cuvette oximeter, so that during the course of an experiment changes in haemoglobin concentration and oxygen saturation of the blood issuing from the submaxillary vein could be followed. A flowmeter connected with the salivary duct, and recording on a Sanborn Polyviso, gave a continuous record of the rate of saliva flow. The resting blood flow was 0.26 ± 0.11 ml/g min and the resting oxygen consumption, 27 ± 6 μl/g min. During activity there was an approximately linear relationship between the rate of saliva flow and the the extra oxygen consumption. There was poor correlation between oxygen consumption and blood flow. Atropine had little or no effect on resting glands, but depressed both the rate of saliva flow and the extra oxygen consumption associated with it, in doses which had no effect on the vasodilator response to stimulation.

Introduction

The correlation between the increases in oxygen consumption and in blood flow, and the secretion of saliva in the stimulated submaxillary gland, was studied by Barcroft and his collaborators (3, 4, 6), but there has been no investigation of the relationship between these three responses in the same gland at different levels of secretory activity. The development of rapid methods of estimation of the haemoglobin concentration and oxygen saturation of blood by oximetry has now made possible a more detailed study of the responses of the gland to stimulation, and of the problem of the atropine-resistant vasodilation. Barcroft found that the vasodilation persisting after secretion had been inhibited by atropine was accompanied by increases in oxygen consumption, though these were smaller than in secreting glands. There is no evidence for non-cholinergic fibers in the chorda and the fact that the vasodilator response can be abolished by heavy dosage with atropine is consonant with a cholinergic initiation of the response.

Methods

The preparation of the submaxillary gland for stimulation and collection of saliva and venous blood was identical with that described previously (9). The dogs were given heparin 3–7 mg/kg to prevent clotting. Oxygen saturation and haemoglobin concentration measurements were made in the cuvette oximeter described by Sekelj and Johnson (19). The accuracy of this instrument is $\pm$ 1.9% of the oxygen saturation in the range 26–100% saturation and $\pm$ 0.5 g% for the haemoglobin concentration.

[1]Manuscript received July 3, 1958.
Contribution from the Department of Physiology, McGill University, Montreal, Que.

The oximeter was arranged so that either arterial samples from a cannula in the central end of the thyroid artery or samples from the venous outflow from the gland could be analyzed as desired. The venous and arterial sampling cannulae were connected to the oximeter inlet by thin polyethylene tubing.

Since the oximeter required a sample of 1.5 ml of blood in 20–30 seconds, special arrangements were required for supplying the venous samples from the resting gland since the flow was in some animals only about 1 ml/min. The device illustrated in Fig. 1 (*a*) was used. The reservoir contained approximately 2 ml of blood, and when the oximeter was operating, its blood requirements in excess of those which could be supplied by the concurrent blood flow were drawn out of the rubber reservoir. This meant that at blood flow rates below 3 ml/min for the whole gland, a proportion of the blood analyzed represented blood that had left the gland shortly before sampling was begun and that had been stored in the reservoir. No appreciable error was introduced

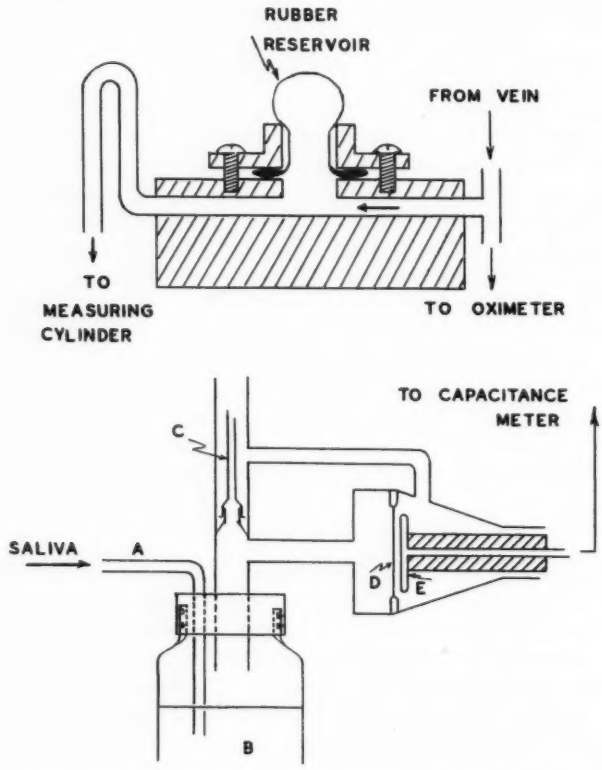

FIG. 1. (*a*) Upper figure. The venous reservoir. (*b*) Lower figure. The flowmeter. A, inlet tube for saliva; B, collecting bottle; the inflowing saliva displaces air through the hypodermic needle, C; D, flexible metal plate; E, fixed metal plate.

by this procedure as the blood was kept out of contact with the air and the reservoir was needed only during the resting periods in which both oxygen saturation and haemoglobin content were constant or changing only quite slowly. Because of the sampling time of the oximeter, the values on blood samples obtained at high flow rates represent approximately the mean values in the 20–30 second sampling time. All equipment in contact with the blood was coated with silicone (Siliclad, Clay Adams).

The effluent from the venous collection device together with the venous samples ejected by the oximeter was collected in a measuring cylinder for estimation of the venous blood flow.

Saliva flow was recorded continuously by a capacitance flowmeter, schematically illustrated in Fig. 1 (*b*). Saliva flows into the bottle and the displaced air leaves the bottle through the hypodermic needle, which acts as a resistance. The pressure drop across the needle is measured by a condenser manometer (Lilly manometer, Technitrol Co., Philadelphia). The maximum pressure drop is less than 1 mm Hg. The capacitance changes are measured on a Fielden proximity meter and recorded on a Sanborn Polyviso. The frequency response of the system cuts off at about 10 cycles/sec and depends on the volume of air enclosed between the surface of the fluid in the bottle and the resistance.

The stimulation periods varied in duration between four and seven minutes in different experiments. Maximal stimuli of 1 millisecond duration were used. The rate of saliva flow was altered by changing the frequency of stimulation. In most of the experiments a constant frequency was maintained throughout a stimulation period; in two experiments saliva flow was kept at a steady rate close to the initial peak rate by progressively increasing the frequency of stimuli during each period.

Arterial blood flow to the gland was calculated from the venous flow rate and the ratio of the haemoglobin concentrations in the arterial and venous blood i.e.

$$ABF = (VBF \times Hb_V)/Hb_A.$$

Oxygen consumption was calculated as follows:

$$\text{Oxygen consumption} = ABF \times O_2 \text{ content arterial blood} - VBF \times O_2 \text{ content venous blood.}$$

Owing to the errors due to dead space and the relatively long sampling period of the oximeter, no reliance was placed on values obtained when the haemoglobin content and oxygen saturation of the venous blood were changing rapidly.

In many experiments the rate of salivary secretion, after the initial peak, became constant during the remaining two to five minutes of stimulation. In others, it fell off very slowly. In contrast to this stabilization of the rate of secretion, the oxygen consumption usually rose during this period. These increases were greatest in stimulation periods where the gland had a high initial peak rate of secretion, or when the saliva flow stabilized at a high rate.

In a few experiments when secretory activity was low, oxygen consumption fell off during stimulation. For this reason, rates of oxygen consumption reported in the results are the mean of the rates during the period of steady saliva flow.

Results

Resting Glands

The results of 122 determinations of blood flow and 100 of oxygen consumption in seven glands, each in a different dog, at rest, are summarized in Table I. Related values from the literature are included. There was considerable variation between different animals and even between values obtained from the same gland at different times. The values obtained in these experiments agree well with those recorded in the literature. No correlation was found between resting oxygen consumption and blood flow. This independence is in agreement with the observations of Barcroft and Muller (5) in the submaxillary gland of the cat.

TABLE I

Blood flow and oxygen consumption in the resting submaxillary gland of the dog

	Blood flow		Oxygen consumption	
Expt.	No. determinations	Mean: ml/g/min (range bracketed)	No. determinations	Mean: μl/g/min (range bracketed)
1	15	0.16±0.04 (0.10–0.21)	12	23±7 (14–33)
2	25	0.30±0.06 (0.19–0.47)	24	23±4) (15–37)
3	26	0.29±0.06 (0.20–0.42)	18	27±4 (15–37)
4	18	0.27±0.05 (0.20–0.40)	7	36±10 (24–48)
5	16	0.20±0.02 (0.17–0.25)	16	31±5 (22–38)
6	6	0.16±0.03 (0.13–0.20)	6	19±3 (14–23)
7	16	0.47±0.07 (0.40–0.57)	16	31±3 (28–38)
Mean, 7 glands		0.26±0.11		27±6
Mean, omitting No. 7		0.23±0.08		
Barcroft and Kato (4) 4 glands		0.20 (0.15–0.27)	7 glands	22 (10–27)
Anrep and Cannan (1) mean, 1 gland		0.25 (0.23–0.29)		
Deutsch and Raper (11) slices		—		21–35
Brock, Druckrey, and Herken (7) slices, —dog, cats, and rats		—		16–32
Stromblad (20) chopped glands, cats		—		32±9

In four experiments the effect of atropine was tested. In two of these the final dose was sufficient not only to abolish the secretion but to reduce the vasodilator response to stimulation. Resting blood flow and oxygen consumption were not affected.

The haemoglobin concentrations in arterial and venous blood in resting glands were the same. The lymph flow must therefore have been less than 10 μl/g min.

Changes during Activity

When the chorda tympani was stimulated, secretion of saliva started after a very brief latency and rose to a peak rate in the first 30–40 seconds of stimulation. Increases in blood flow were considerable within the first twenty seconds. The first oximeter samples were taken 30 seconds after the start of stimulation, and showed within this period increased haemoglobin concentration and oxygen saturation of the venous blood (Fig. 2). At the end of

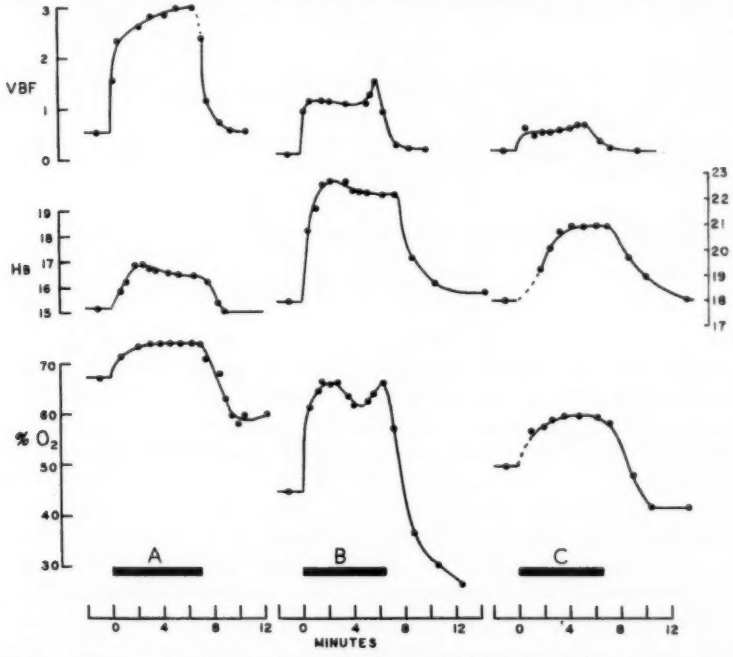

FIG. 2. Changes in the rate of flow, the haemoglobin concentration, and the oxygen saturation of venous blood during stimulation periods indicated by black bars. The examples are from experiments in which a steady rate of secretion was maintained by progressively increasing the frequency of stimulation during the secretion period.

Ordinates: VBF, venous blood flow, ml/g/min; Hb, haemoglobin concentration, g/100 ml blood; on left, for A; on right, for B and C; % O_2, oxygen saturation of the venous blood.

Arterial O_2 saturation A, 93%; B and C, 95%. A. Stimulation at 8.75–12 c.p.s. Saliva flow, 380 μl/g/min. B. Stimulation of a different gland at 11–16 c.p.s. Saliva flow, 370 μl/g/min. C. Stimulation of the same gland as in B at 1.8–2.5 c.p.s. Saliva flow, 75 μl/g/min.

stimulation periods, secretion stopped immediately and blood flow and oxygen saturation fell rapidly towards their resting values, which they reached in from two to ten minutes. The oxygen saturation of the venous blood fell to below its resting level temporarily after a stimulation period, while at the same time the oxygen consumption of the gland remained elevated above its resting rate.

In Fig. 3 are plotted the concurrent changes in the rates of saliva secretion, arterial blood flow, and oxygen consumption during three stimulation periods in two glands, illustrating representative patterns of response. After stimulation had ceased there was a brief period of oxygen debt, lasting from one to eight minutes and amounting to only a few per cent of the increased oxygen consumption during the secretory period. The increased oxygen consumption during stimulation was graded according to the rate of salivary secretion.

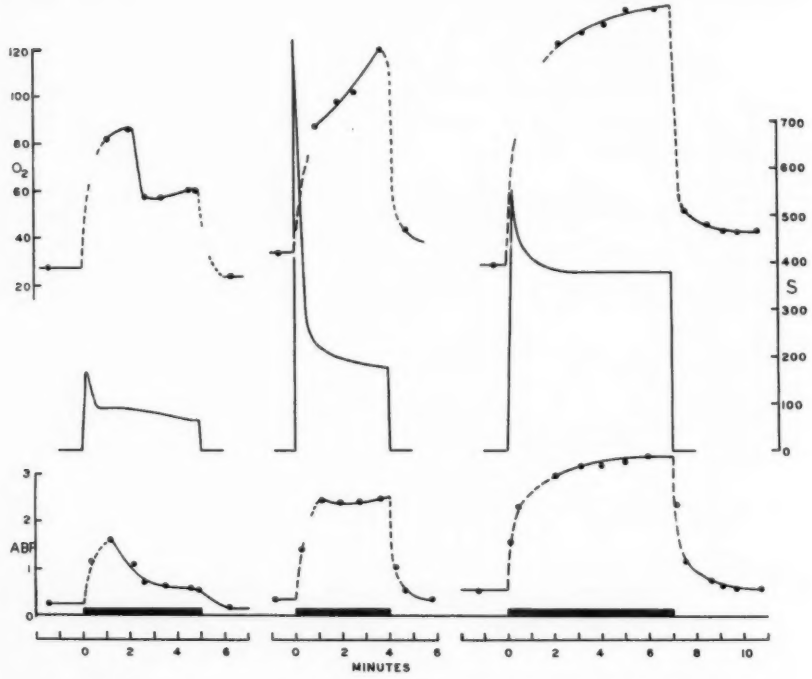

FIG. 3. Typical responses to chorda stimulation, indicated by the black bar. Ordinates from above downwards: O_2 = O_2 consumption, μl/g/min; S = saliva flow, μl/g/min; ABF = arterial blood flow, ml/g/min. The open circles are values for venous blood flow.

From left to right: stimulation at 2 c.p.s.; stimulation of the same gland at 20 c.p.s.; stimulation of a different gland, where the frequency of stimulation was gradually increased from 8.75 to 12 c.p.s. during the stimulation period to maintain a high rate of saliva flow. (Interrupted lines in these and other figures show the direction of the changes during periods where determinations of the haemoglobin concentration and oxygen saturation of the venous blood are considered unreliable because of rapidly changing conditions.)

In Fig. 4 the rates of saliva secretion in the stable periods are plotted against the corresponding mean rates of extra oxygen consumption. At the maximal steady rate of saliva secretion (380 μl/g min), the excess oxygen consumption was about 100 μl/g min, as compared with the average resting value of 27 μl/g min.

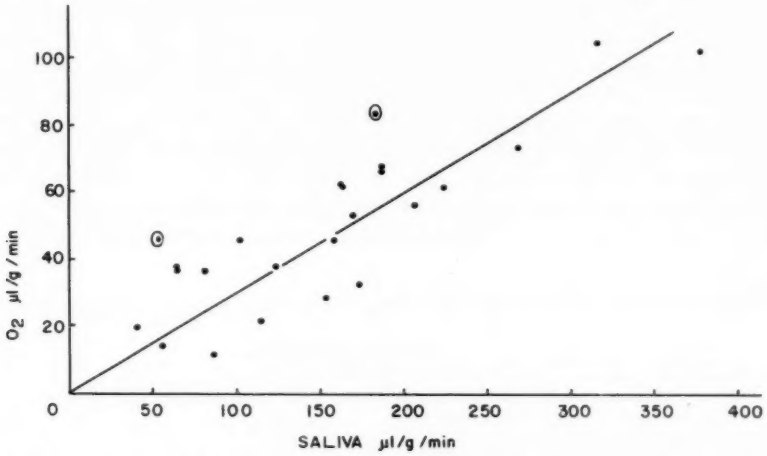

FIG. 4. The relation between the rate of saliva flow and the extra oxygen used during 24 periods of secretory activity in seven glands. The two circled points are two values for a gland which showed an exceptionally great increase in oxygen consumption during activity. Line drawn by eye disregarding the circled points.

The extra oxygen consumption was linearly related to the rate of saliva secretion so that the production of each milliliter of saliva was associated with the utilization of 0.3 ml of oxygen. Using the approximate equivalence of 1 ml oxygen = 5 calories, the metabolic work associated with the secretion of saliva was about 1500 cal/l. This may be compared with the osmotic work done in the formation of parotid saliva (8), which at low rates of secretion reached a maximum of 105 cal/l. At high rates of secretion the work fell to as little as 12 cal/l. The thermodynamic osmotic work of formation thus gives little indication of the metabolic work entailed in the secretion of saliva.

As in the resting state, there was little correlation between the increase in oxygen consumption and the increased blood flow through the gland (Fig. 5).

The Effect of Atropine on Activity

The dogs varied in their sensitivity to atropine. Four hundred micrograms per kilogram depressed but did not abolish secretion in one, while at the other extreme 36 μg/kg suppressed secretion for half an hour. Recovery of the secretory response to stimulation was followed in one of these dogs as the effects of atropine wore off.

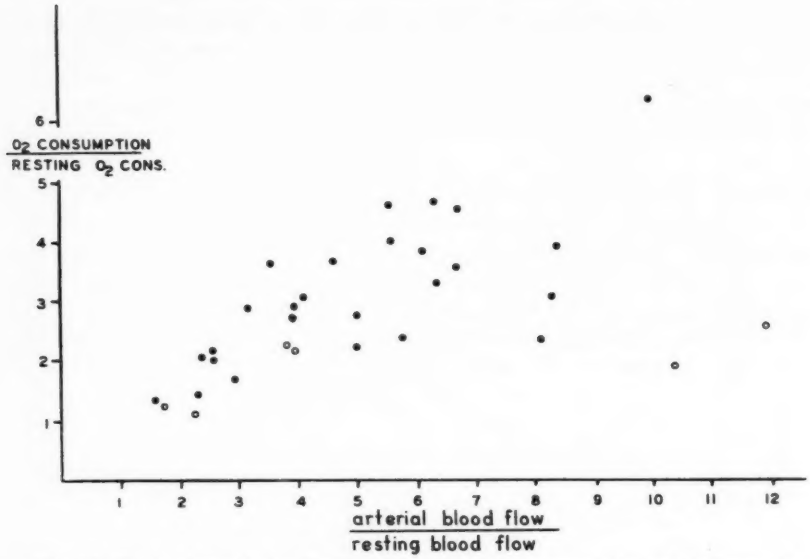

FIG. 5. Lack of correlation between increases in O_2 consumption and increases in arterial blood flow during secretory activity.
● Before atropine, seven glands.
○ After atropine, three glands.

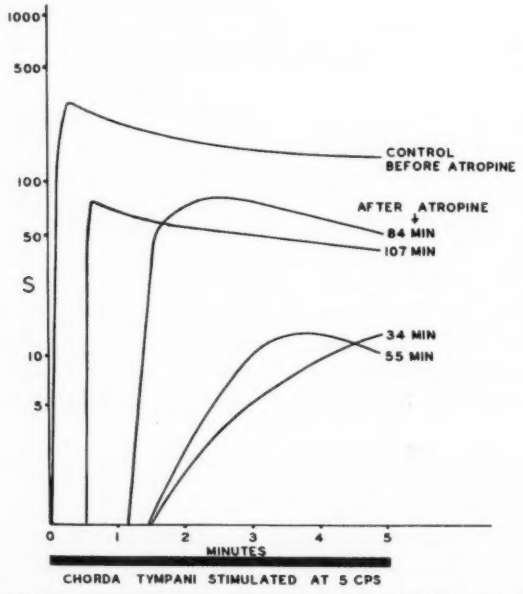

FIG. 6. The delayed onset of secretion in a gland recovering from a dose of atropine of 36 μg/kg, which abolished secretion of saliva for 30 minutes. Ordinate S—saliva flow, μl/g/min, logarithmic scale.

When the dose of atropine was insufficient to abolish secretion, the onset of the reduced secretion was delayed, an effect noticed by Langley in 1898 (17). The latent period shortened as the effect of atropine wore off (Fig. 6). Compared with the response to identical stimulation before atropine, oxygen consumption fell in parallel with the decrease in secretion rate, while the increased blood flow was as great or greater than before (Fig. 7). There were no exceptions to this dissociation of the secretory and the vasodilator responses to stimulation in eight separate stimulation periods, in four dogs. When the dose of atropine was sufficient to abolish secretion, oxygen consumption during stimulation was further reduced, so that an average increase of only 21% in five stimulation periods occurred compared to 230% with the same stimulation before atropine. Still the same or a greater increase in blood flow occurred. This enhanced effect of stimulation on the blood flow after atropine can probably be explained by the lack of haemoconcentration in the blood passing through the gland when no secretion is occurring.

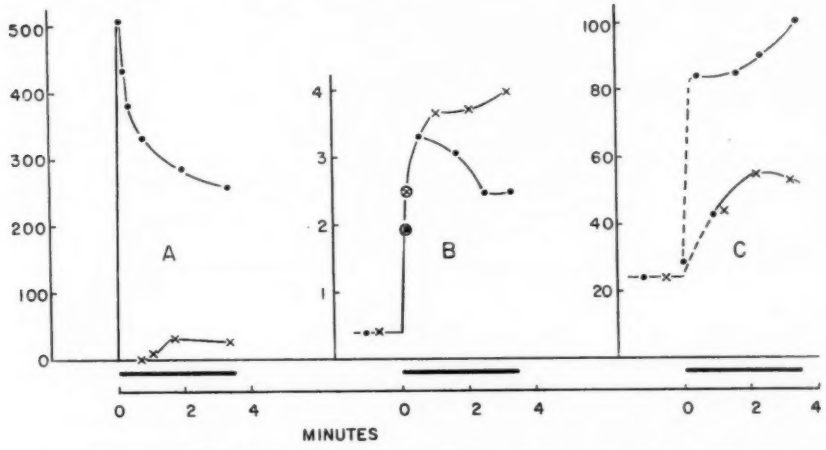

FIG. 7. From left to right: simultaneous saliva flow, arterial blood flow, and oxygen consumption in the same gland stimulated at 5 c.p.s. before (●) and after (×) a dose of atropine insufficient to abolish secretion.
Ordinates: A—saliva flow, μl/g/min; B—arterial blood flow, ml/g/min; C—oxygen consumption, μl/g/min.
The circled points are for venous blood flow; arterial flow would be greater.

Increasing the atropine dosage (1 mg/kg in the experiment in Fig. 8) depressed the vasodilator as well as the secretory and metabolic responses to stimulation.

The persistence of full vasodilation after atropine, with secretion depressed or entirely abolished does not fit in with Barcroft's view that the vasodilation is due mainly to secretory metabolites, though these may possibly play a part at high secretory rates. Nor do our results support the conclusion of Hilton and Lewis (13, 14, 15, 16) that secretion, metabolism, and vasodilation go hand in hand. These authors have demonstrated the formation of a

bradykinin-like substance in the submaxillary gland of the cat, which they consider responsible for the vasodilator response to nerve stimulation. In their view the chorda fibers are all identical, and have only one effect on the gland, to stimulate the metabolic response which results in both secretion of saliva and release of the bradykinin-forming enzyme. They attribute vasodilation after atropine, when overt secretion of saliva is suppressed, to a persisting low level of functional activity, basing their evidence for this persistence on experiments of Barcroft (3) in which he found increased oxygen consumption and vasodilation after atropine sufficient to abolish the secretion, and of Anrep and Cannan (2), who found increased glucose utilization and vasodilation without secretion, but the latter authors also report a ninefold increase in blood flow after atropine without increased glucose uptake or secretion of saliva.

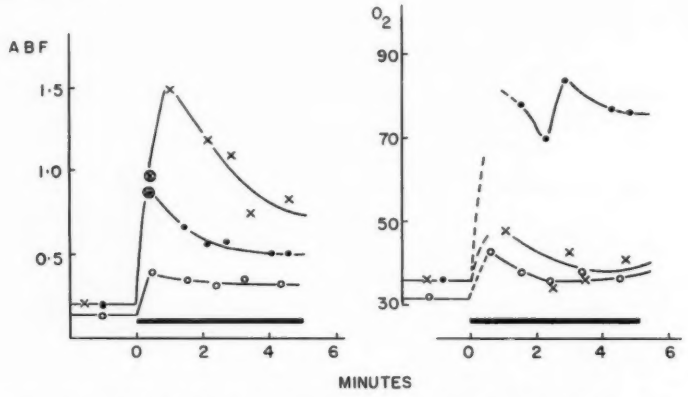

FIG. 8. Arterial blood flow and oxygen consumption in a gland before and after a dose of atropine sufficient to abolish secretion. Stimulation at 5 c.p.s.
Ordinates: ABF, ml/g min; O_2 consumption, μl/g/min.
●———● Before atropine. Saliva flow 60 μl/g/min after an initial peak rate of 106 μl/g/min.
×———× 15 minutes after 1 mg/kg atropine. No secretion.
○———○ 35 minutes after the same dose of atropine. No secretion.

Our results show that the vasodilator responses of the gland are not secondary to the secretory metabolism. The metabolic and vasodilator responses can be dissociated with atropine, showing that atropine can preferentially enter those receptor sites in the structure of the gland associated with the secretory response. Dale and Gaddum (10) came to the same conclusion.

The metabolic pattern of the secreting salivary gland has been studied by several workers. Anrep and Cannan (1, 2) found that the glucose utilization of the submaxillary gland of the dog was correlated with the rate of secretion of the saliva. Northrup (18) found a depletion of creatine phosphate and glycogen in the submaxillary gland of the cat after prolonged stimulation together with an increase in the lactic acid content. Deutsch and Raper (12) also found some lactic acid production to occur when submaxillary gland

slices were stimulated with acetylcholine. These results suggest that part of the energy metabolism of the secreting gland occurs through anaerobic glycolysis.

Acknowledgment

This work was generously supported by a grant from the National Research Council of Canada.

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SOME PHYSIOLOGIC CHARACTERISTICS OF ESTERS OF CETYL ALCOHOL[1]

DORIS HOWES CALLOWAY, GEORGE W. KURTZ, AND ROBERT B. POTTS

Abstract

Palmitic, oleic, and butyric acid esters of cetyl alcohol were fed to mature rats. Cetyl palmitate was quantitatively excreted in the feces and produced no physiologic aberration. While cetyl oleate was 75% absorbable, it was of little value as an energy source and was partially excreted through the skin. Feeding of cetyl butyrate also induced seborrhea. Data on free fatty acids in gastrointestinal contents and on the specificity of seborrheic lipids indicate that cetyl esters are largely absorbed without hydrolysis, if the fatty acid moiety is readily absorbable, and excreted by way of the skin without modification.

Introduction

In a search for high melting point lipids showing sufficient digestibility to permit incorporation into specialized Armed Forces rations, the nutritive properties of fatty acid esters of long chain, monohydric alcohols were selected for investigation. Previous studies indicated that in the case of triglyceride lipids and fatty acid esters of the hexahydric alcohol mannitol digestibility was a function of the chain length of the component saturated fatty acids rather than of melting point per se (1). A possible technique for increasing melting point without recourse to hydrogenation consists of further increasing the chain length of the component alcohol. Blomstrand and Rumpf (2) reported that approximately 50% of an administered dose of C^{14}-labelled cetyl alcohol was incorporated into lymphatic triglyceride after oxidation to palmitic acid in the intestinal mucosa. Accordingly, oleic and palmitic acid esters of cetyl alcohol were prepared and evaluated as potential energy sources.

Experimental

Preparation of Materials

Cetyl alcohol (1.03 moles) and 1.00 mole of fatty acid were dissolved in three times their volume of benzene and heated under reflux. Approximately 3% of sulphuric acid, based on the weight of the alcohol, was used to catalyze the reaction. After 4 hours of reflux the mixture was cooled and washed with 1.00 mole of sodium hydroxide in approximately 0.1 *N* solution. The benzene extract was then filtered and benzene removed by vacuum distillation. The ester was separated by molecular distillation twice. The only impurity detected by infrared spectroscopy was a small amount of cetyl elaidate in the

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cetyl oleate preparation. Capillary melting point, iodine number, saponification and thiocyanogen values were determined (3) and showed good agreement with published or theoretical values (see Table I).

Part I

In the first test cetyl oleate and cetyl palmitate were incorporated as 20% by weight of a fully adequate purified diet (1). These diets, and others that were fat-free or contained 20% cottonseed oil (control diets), were fed to groups of five male albino rats (body weight 200–250 g) for 9 days. The animals were given 12 g of diet per day and water ad libitum. Body weights were recorded every third day and food consumption daily. Feces were collected over the last five days for measurement of fat absorption. Lipid content of the diet and of the feces was determined by a modified procedure of the Association of Official Agricultural Chemists (1). Lipid absorbability was calculated from the formula:

$$\text{Absorbability } \% = \frac{\text{lipid intake} - (\text{fecal lipid} - \text{fecal lipid of group fed none}) \times 100}{\text{lipid intake}}.$$

Three additional groups were given diets providing 20% of equal mixtures of the following lipid materials: (*a*) cetyl alcohol plus oleic acid, (*b*) cottonseed oil plus cetyl alcohol, or (*c*) cottonseed oil plus oleic acid. Fat absorption was not measured in these groups.

TABLE I

Characteristics of lipids

Measure	Cotton-seed oil	Cetyl oleate		Cetyl palmitate		Cetyl butyrate	
		Obs.	Theor.	Obs.	Theor.	Obs.	Theor.
Capillary m.p., ° C	<0	27.5		52.1		12.9	
Iodine number	113	51	50.1	<1	0	<1	0
Thiocyanogen value	69	46	50.1	0	0	1	0
Saponification value	196	114	110.7	117	116.7	180	179.5
Absorbability, %	96	75		0			

Absorption of cetyl oleate was found to be 75.3% of the amount fed but the cetyl palmitate was recovered quantitatively in the feces (see Table I). Absorption of the cottonseed oil was 95.9%. During the 9-day test period the group fed cottonseed oil gained an average of 21 g in body weight on a mean intake of 480 absorbable calories. The groups given cetyl palmitate and cetyl oleate lost 3 and 4 g on intakes of 301 and 371 cal of absorbable material, respectively. These computed data indicate that only a small portion of the absorbed cetyl oleate was utilizable as an energy source.

After 1 day of feeding, animals given cetyl oleate developed seborrhea which progressively increased as feeding continued (see Figs. 1 and 2). The seborrhea began at the dorsal base of the tail and spread over the entire

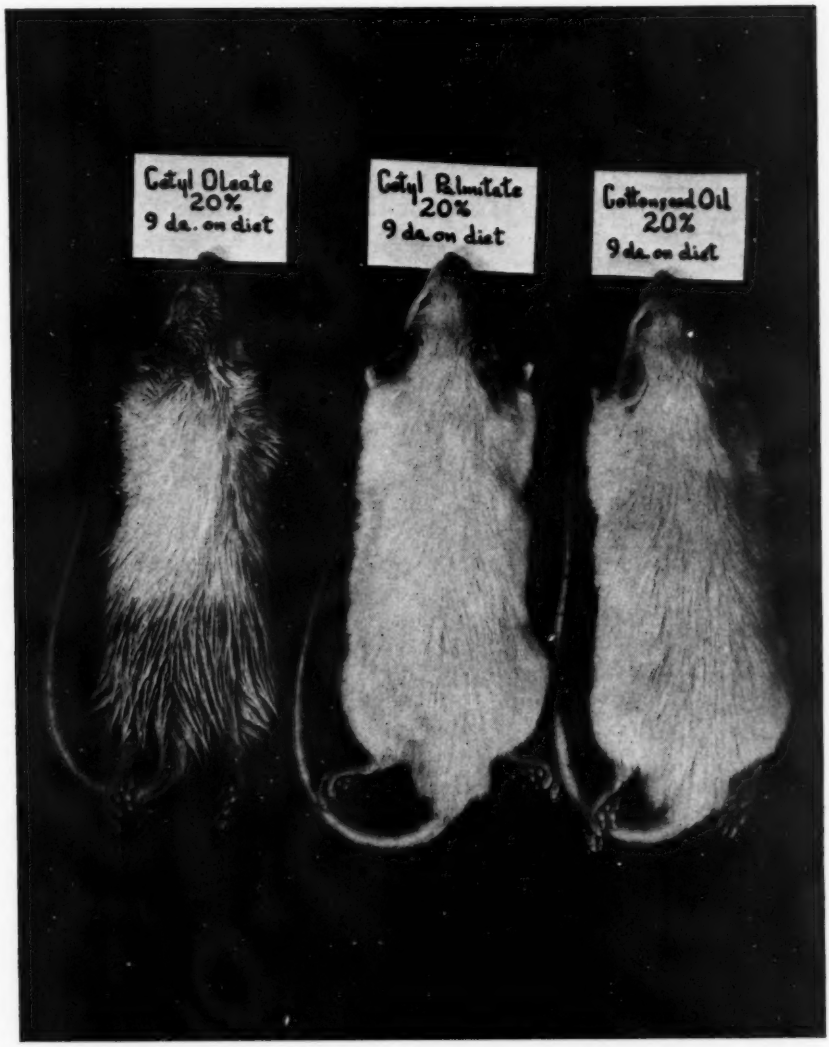

FIG. 1. Dorsal view of rat fed cetyl oleate showing seborrhea and normal appearance of rats fed cetyl palmitate or cottonseed oil.

FIG. 2. Ventral view of rat fed cetyl oleate showing seborrhea and normal appearance of rats fed cetyl palmitate or cottonseed oil.

ventral surface, feet, and head. The median area of the back was least affected. Post-mortem examination revealed thickening and hyperemia of the intestinal wall. Infrared spectroscopy of a sample of the exuded lipid showed identity with the fed compound. Animals given cetyl palmitate, cetyl alcohol, oleic acid, or a simple mixture of the latter two exhibited no seborrhea or intestinal abnormality.

Part II

The absorbability and seborrhea findings suggested that fatty acid esters of cetyl alcohol were not hydrolyzed in the gut but were absorbed as the ester if the fatty acid component were facilitating. Secondly, it appeared that the absorbed compound was virtually non-utilizable as an energy source being excreted by the skin. To test these hypotheses cetyl esters of a short chain saturated fatty acid (butyric) as well as of the mono-unsaturated, long chain oleic acid were studied. These compounds were fed alone and in conjunction with triglycerides. To detect lipolysis the free fatty acid content of chyme was measured as indicated. Ester rearrangement should be detectable, if it occurred, by finding, in hair lipids, cetyl oleate when a mixture of cetyl butyrate and triolein was fed or vice versa.

Cetyl oleate, cetyl butyrate, and equal mixtures of cetyl oleate plus tributyrin and cetyl butyrate plus triolein were administered by stomach tube. The groups of six male rats (body weight 350 to 459 g) were given 2 g of lipid daily* and free access to a fat-free diet. Control animals were intubated with 2 ml of 40% sucrose solution. On the 10th day the animals were fasted overnight and two animals per group sacrificed; the remaining animals were sacrificed at 1 and 3 hours after administration of 2 g of lipid* or sucrose. At sacrifice the stomach and 120 cm of small intestine were removed and the contents washed out with normal saline. The intestine was blotted and weighed. The free fatty acid content of gastric and intestinal contents was measured by titration with alcoholic KOH after separation from mineral acid by column chromatography (4). A sample of dorsal skin with hair was obtained and hair lipids extracted with carbon tetrachloride. The infrared absorption pattern of this material was determined.

Earlier onset of severe seborrhea was noted with cetyl butyrate than with cetyl oleate. In both groups the infrared pattern of exuded lipid was identical with the starting material including the presence of cetyl elaidate. When the mixture of cetyl oleate and tributyrin was fed only cetyl oleate was recovered, indicating that no ester rearrangement had occurred. In animals given the mixture of cetyl butyrate and triolein neither compound was recovered in significant amount, the pattern being characteristic of normal hair lipids. This failure may be ascribable to the lower dosage which these animals received.

Intestinal weight was markedly increased in the groups given cetyl preparations except in the presence of tributyrin (see Table II). The increased

*Except in the case of cetyl butyrate plus triolein where limitations of materials necessitated the use of only 0.5 g of each.

weight was not associated with deposition of the test materials in the intestinal wall. The most probable variation was in moisture content, based on the edematous, hyperemic appearance of the tissue.

TABLE II

Free fatty acids in gastric and intestinal contents

Lipid	Dose, g/day	Wt. of 120 cm small intestine,* g	Free fatty acids of contents, μeq above control			
			Stomach		Small intestine	
			+ 1 hr	+ 3 hr	+ 1 hr	+ 3 hr
None	0	5.85 (5.59–6.08)				
Cetyl oleate	2.0	8.25 (7.80–8.55)	NSD†	2.0	7.0	3.4
Cetyl butyrate	2.0	9.56 (9.00–11.17)	NSD	NSD	NSD	NSD
Cetyl oleate plus tributyrin	1.0 + 1.0	6.15 (5.96–6.41)	5.8	5.9	2.6	NSD
Cetyl butyrate plus triolein	0.5 + 0.5	8.77 (8.12–9.48)	6.0	12.6	3.6	NSD

*Mean and range.
†No significant difference.

In evaluation of free fatty acid release in gut contents it was assumed that mild acid hydrolysis would be detectable by the presence of free fatty acids in stomach contents and enzymic hydrolysis by the detection of these acids in intestinal material. Very low recovery could be expected in intestinal samples because of the simultaneous absorption of freed acid. As shown in Table II, no hydrolysis of cetyl butyrate was observed at any time interval in any sample. Minor but delayed hydrolysis of cetyl oleate was evidenced by the presence of free fatty acid in the 3-hour gastric sample and 1-and 3-hour intestinal samples. When triglycerides were fed, free fatty acids were found in all samples except the 3-hour intestinal contents when it would appear that absorption was keeping pace. These data indicate that cetyl esters are hydrolyzed with difficulty if at all.

Discussion

The seborrhea-producing property of sperm and bottlenose whale oils was reported by Somekawa in 1933 (5). This investigator concluded after an extensive series of tests (6) that the seborrhea-inducing agent was oleyl oleate and that the position of the double unsaturation was influential as geranyl palmitate, cetyl oleate, and cetyl linoleate were ineffective, in divergence with the findings reported here. The seborrhea-producing quality of the oil was found to be destroyed by hydrogenation and oxidation leading the author

to conclude a parallelism with iodine value (7). Our evidence indicates that saturation is not of itself the determining factor, fully saturated cetyl butyrate proving as active as the mono-unsaturated cetyl oleate. It would appear that those fatty acids which facilitate absorption in the case of triglycerides, i.e., short-chain saturates and long-chain unsaturates (1), are equally effective in enhancing absorbability of the long-chain alcohol.

Of particular interest is the observation that the cetyl esters were absorbed with little apparent lipolysis when solubility characteristics permitted or were quantitatively excreted. Somekawa also found that when the seborrhea-producing diet contained fat soluble pigments, these diffused from the skin (6). The use of such compounds for the direct transmission of materials to the skin is worthy of consideration in therapy.

Acknowledgment

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STUDIES OF LIGNIN BIOSYNTHESIS USING ISOTOPIC CARBON

VII. THE ROLE OF *p*-HYDROXYPHENYLPYRUVIC ACID[1]

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Abstract

L-Phenylalanine-C^{14} and *p*-hydroxyphenylpyruvic acid-3-C^{14} were administered to wheat, buckwheat, and salvia (*Salvia splendens* Sello), and after 24 hours the "lignin aldehydes" (*p*-hydroxybenzaldehyde, vanillin, and syringaldehyde) were isolated following alkaline nitrobenzene oxidation of the plant fraction insoluble in ethanol–benzene and water. Although all species converted phenylalanine readily to lignin, only wheat could form the syringyl and guaiacyl parts of lignin efficiently from *p*-hydroxyphenylpyruvic acid. *p*-Hydroxybenzaldehyde recovered from all species was heavily labelled after feeding both compounds. The L-tyrosine from acid hydrolysis and the *p*-hydroxybenzaldehyde from nitrobenzene oxidation, isolated from buckwheat activated by feeding *p*-hydroxyphenylpyruvic acid-3-C^{14}, had the same molar specific activity, and it is considered probable that most if not all of the *p*-hydroxybenzaldehyde in all these species was derived from protein tyrosine in the extracted residue. When *p*-hydroxyphenyllactic acid-3-C^{14} was metabolized by wheat all the aldehydes were labelled, but none possessed measurable radioactivity when this compound was administered to buckwheat. Consequently, neither *p*-hydroxyphenylpyruvic nor *p*-hydroxyphenyllactic acid is a general intermediate in lignification, and differences noted here and in previous papers between grasses and non-grasses probably result from the unique ability of grasses to convert *p*-hydroxyphenyllactic acid to *p*-hydroxycinnamic acid. This idea is supported by the ability of wheat to form *p*-hydroxycinnamic and ferulic acids readily from both phenylalanine and tyrosine, whereas only phenylalanine is a good precursor of these acids in salvia. A scheme is presented showing the metabolic interconversions of phenylpropanoid acids and related compounds leading to lignin.

Introduction

The function of phenylalanine as an efficient lignin precursor has been firmly established in all plant species thus far studied (1, 2, 3). In earlier publications of the present series it has been determined that in favorable cases phenylalanine can be readily converted, with dilutions of C^{14} lower than 50, into the three types of lignin monomer, as represented by the lignin oxidation products: *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde. In contrast, the utilization of tyrosine in lignification is subject to a marked species variation, and among 12 species representing 10 families investigated, only 2 members of the grass family were able to convert this compound as efficiently as L-phenylalanine to guaiacyl and syringyl lignin residues. In other species tyrosine was less efficiently utilized by a factor of about 100.

This led two of us (2) to suggest that the grasses possess a tyrosine-metabolizing enzyme not present in the other species tested. As *p*-hydroxyphenylpyruvic acid is a logical intermediate between tyrosine and the substituted cinnamyl alcohols believed (4) to be the lignin monomers, it was further

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[2]National Research Council Postdoctorate Fellow, 1957–1958.

suggested that the block in these other species may arise through the absence of a tyrosine transaminase or oxidase, or through the lack of an enzyme required to convert *p*-hydroxyphenylpyruvic acid to the lignin monomers. In the second event *p*-hydroxyphenylpyruvic acid, too, would not be convertible to lignin in plants other than grasses.

Recently, Acerbo, Schubert, and Nord (5) have reported that *p*-hydroxyphenylpyruvic acid is converted into lignin by sugar cane over a 15-day period. Although no comparative data were obtained for other precursors under these conditions of feeding, the dilution of carbon-14 in the reactions, calculated on the basis of an average of 10.5 carbons per unit in the lignin polymer, is about 30, which suggests that this precursor is efficiently utilized. In view of the fact that sugar cane is a grass, this result is not unexpected. These authors, who apparently lacked access to earlier findings from our laboratory, have drawn the conclusion that *p*-hydroxyphenylpyruvic acid is a precursor of lignin "building stones", without any qualification concerning possible species differences.

In order to shed further light on the comparative biochemistry of the interconversions of C_6,C_3 compounds in plants, and in an attempt to resolve the question about the function of *p*-hydroxyphenylpyruvic acid in lignification, metabolic studies of this and related compounds were undertaken.

Experimental

Cultivation of the Plants

Wheat (*Triticum vulgare* Vill. var. Thatcher), salvia (*Salvia splendens* Sello var. Globe of Fire), and buckwheat (*Fagopyrum tataricum* Gaertn. var. C.D. 4521) were grown in gravel culture in a growth chamber under "cool white" fluorescent and incandescent illumination giving a combined intensity of 13,000 lumens/m² (1200 ft-c) and a day length of 18 hours. The experiments were conducted during the early flowering stages.

Synthesis of C^{14}-Labelled Compounds

p-Hydroxybenzaldehyde-carbonyl-C^{14} (2) was converted to p-*hydroxyphenylpyruvic acid-3-C^{14}* by condensation with acetylglycine, as described by Saul and Trikojus (6). A portion of this keto acid was reduced to DL-p-*hydroxyphenyllactic acid-3-C^{14}* with sodium amalgam (6).

Randomly labelled L-phenylalanine and L-tyrosine were purchased from Atomic Energy of Canada, Ltd., Ottawa.

Administration of Labelled Compounds

L-Phenylalanine, L-tyrosine, and the sodium salts of *p*-hydroxyphenylpyruvic and *p*-hydroxyphenyllactic acids were administered through the cut ends of the stems in aqueous solution, essentially as described earlier (2). After the absorption of the radioactive solution, the plants were allowed to metabolize in a chamber illuminated by fluorescent tubes with an intensity of *ca.* 5000 lumens/m² (500 ft-c) for approximately 24 hours at 20° C.

Isolation of Lignin Derivatives

The preparation and oxidation of the cell wall fraction, and the isolation and purification of the phenolic aldehydes were done as described previously. The aldehydes were separated on columns of diatomaceous earth, and converted to their *m*-nitrobenzoylhydrazones (1).

Isolation of Organic Acids

The isolation of *p*-hydroxycinnamic and ferulic acids from wheat was carried out by the procedure of McCalla (7). Sinapic acid was also present in the extract, as identified by the characteristic fluorescence and R_f value on a paper chromatogram developed with toluene – acetic acid – water, but in this instance it could not be satisfactorily purified.

Isolation of Tyrosine

Buckwheat plants were cut into small pieces with scissors, dried *in vacuo* at 60° C, ground to pass a 60-mesh screen, and then extracted successively with hot ethanol–benzene (1+2), and hot water, in a continuous extractor. A 0.7-g sample of the residue from these treatments was heated in the steam bath with 15 ml of 6 *N* hydrochloric acid for 24 hours in a sealed tube. The reaction mixture was filtered and the filtrate evaporated on a steam bath under a current of air until no odor of hydrochloric acid was detectable.

The residue was taken up in water, filtered free of insoluble material, and the filtrate passed through a 1×9 cm column of IR-120-H cation-exchange resin. The material retained on the column, including the amino acids, was displaced with *N* ammonium hydroxide. The eluate, concentrated to 1 ml, was pipetted on the top of a 1.2×26 cm column of Dowex 1-X8 resin, prepared according to Hirs, Moore, and Stein (8). The column was developed with 0.5 *N* acetic acid at the rate of 6 ml per hour, 2-ml fractions being collected. Tyrosine, located by spot tests with a phenol reagent (1), appeared in fractions 12 to 17.

The solution obtained by combination of these fractions was analyzed for tyrosine by the colorimetric procedure of Ceriotti and Spandrio (9). After the addition of an appropriate amount of carrier, the tyrosine was recrystallized from water to constant specific activity.

Counting of Carbon-14

The organic compounds were wet-combusted to carbon dioxide, which was counted in the gas phase essentially as described earlier (1).

Results and Discussion

In the present experiments the plants tested were wheat, which, like sugar cane, is a member of the Gramineae, and buckwheat and salvia, two non-grasses of widely differing phylogeny. The results of experiment 1 in Table I show that *p*-hydroxyphenylpyruvic acid was apparently utilized by wheat about twice as efficiently as phenylalanine for the formation of *p*-hydroxyphenyl lignin, and about one-quarter as efficiently for the synthesis of the other two

TABLE I

Utilization of *p*-hydroxyphenylpyruvic and *p*-hydroxyphenyllactic acid for lignin formation

Species	Experiment	Compound administered			Specific activity of isolated aldehydes, μc/mM			Dilution* (corr. for plant wt.)		
		Name	Specific activity, μc/mM	Dose, μM/g dry wt.	*p* Hydroxybenzaldehyde	Vanillin	Syringaldehyde	*p*-Hydroxybenzaldehyde	Vanillin	Syringaldehyde
Wheat	1	L-Phenylalanine	32.0	27.7	0.535	0.846	1.1	59.8	37.8	29
		DL-*p*-HPPA	31.9	33.0	1.15	0.229	0.295	33.0	166	129
	2	L-Phenylalanine	42.5	20.3	0.622	0.804	0.885	68.3	52.9	48.0
		DL-*p*-HPLA	27.1	16.9	0.430	0.185	0.314	52.5	122	71.9
Buckwheat	3	L-Phenylalanine	38.9	28.6	0.352	1.78	1.63	111	21.8	23.8
		DL-*p*-HPPA	49.6	25.5	0.643	0.015	0.015	68.8	2900	2900
	4	L-Phenylalanine	48.1	18	0.12	2.72	0.022	400	17.7	2200
		DL-*p*-HPLA	26.1	19	0	0	0	∞	∞	∞
Salvia	5	L-Phenylalanine	25.9	20.8	0.156	0.459	0.460	166	56.4	56.3
		DL-*p*-HPPA	32.9	25.5	1.21	0.006	0.010	33.4	7000	4000

NOTE: HPPA = hydroxyphenylpyruvic acid; HPLA = hydroxyphenyllactic acid.
*Observed dilution equals the value in column 4 divided by that in column 6, 7, or 8.

types of lignin. Insofar as data for tyrosine are available (1) the figures for *p*-hydroxyphenylpyruvic acid and tyrosine are roughly comparable. The other species (experiments 3 and 5) were able to convert the keto acid to guaiacyl and syringyl lignin only with difficulty, utilizing it some 40 to 200 times less efficiently than phenylalanine. Thus, at least part of the deficiency in these two species must concern their ability to utilize *p*-hydroxyphenylpyruvic acid, and it also follows that *p*-hydroxyphenylpyruvic acid is not a lignification intermediate in all species.

Although most non-grasses yield very small amounts of *p*-hydroxybenzaldehyde on nitrobenzene oxidation of the cell wall residue, an effort was made for the first time to recover enough for accurate C^{14}-analysis. This proved successful, and yielded the surprising information that buckwheat and salvia were apparently able to use *p*-hydroxyphenylpyruvic acid as well as, or better than, phenylalanine for the synthesis of *p*-hydroxyphenyl lignin. This finding immediately raised once again the question of the origin of *p*-hydroxybenzaldehyde. In an early communication from these laboratories Stone, Blundell, and Tanner (10) suggested that this compound might arise from the oxidation of tyrosine in the protein associated with the cell wall. They found that DL-tyrosine, oxidized with nitrobenzene under the standard conditions, yielded 16% of the theoretical amount of *p*-hydroxybenzaldehyde, and they presented calculations suggesting that in wheat all the *p*-hydroxybenzaldehyde could be accounted for by protein tyrosine. De Stevens and Nord (11) have shown, though, that nitrogen-free native lignin from another grass—bagasse—does give rise to *p*-hydroxybenzaldehyde.

In an effort to settle the question, *p*-hydroxyphenylpyruvic acid-3-C^{14} was fed to two buckwheat plants as in the first experiment, and the pooled plants were then dried, ground, and extracted with ethanol–benzene and water as before. Of the 3.5 g of extracted residue, 2.8 g was oxidized with nitrobenzene in alkali and the remaining 0.7 g was hydrolyzed with hydrochloric acid for the liberation of protein tyrosine. The dilutions of carbon-14 in the conversion of *p*-hydroxyphenylpyruvic acid to *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde were 62, 690, and 1200, respectively—dilutions somewhat lower than recorded in the earlier experiment. The specific activity of the *p*-hydroxybenzaldehyde was 0.84 μc/mM, whereas that of the tyrosine recovered from the protein hydrolyzate was 0.83. There is no assurance that either recovery was quantitative, and hence yield calculations are not too meaningful, but the best estimate from the recoveries obtained is about a 30% yield of *p*-hydroxybenzaldehyde from tyrosine; this compares with the 16% obtained from a pure sample by Stone *et al.* (10).

It is clear from the specific activities reported above that the carbon-14 recovered in the *p*-hydroxybenzaldehyde can all be accounted for by the oxidation of protein tyrosine, and it is assumed that this also holds true for salvia. *p*-Hydroxyphenylpyruvic acid is thus a good precursor of tyrosine in buckwheat, and it is very likely an intermediate in tyrosine formation from

prearomatic compounds. The conversion of *p*-hydroxyphenylpyruvic acid to tyrosine provides evidence that there is no metabolic block at this stage in lignification, but only beyond the keto acid.

Tyrosine was also estimated in a hydrolyzate of a cell wall residue from wheat. Plants about 50 days old, less the heads, were dried, ground, extracted with ethanol–benzene and with water, and analyzed as described in the Experimental section. A tyrosine content of 0.38% was found, and as the yield of *p*-hydroxybenzaldehyde from similarly treated wheat residue is only about 0.1%, it is evident that here, too, a major part of this aldehyde can be accounted for by oxidation of tyrosine associated with the cell wall material. Clearly, the carbon-14 content of *p*-hydroxybenzaldehyde obtained by oxidation of cell wall residues (or presumably isolated lignins) is a much less reliable criterion of lignin radioactivity than that of vanillin and syringaldehyde, and would be of value only if a cell wall fraction with no significant nitrogen content were used.

An attempt has been made to localize further the metabolic block in non-grasses by a study of the utilization of labelled *p*-hydroxyphenyllactic acid, the expected reduction product of *p*-hydroxyphenylpyruvic acid. Enough labelled compound was available for testing in only two species. In this experiment, wheat was chosen as the grass and buckwheat as the representative of another family. Experiments 2 and 4 of Table I show that in wheat, as anticipated, this compound was utilized efficiently in lignification; in buckwheat, however, it gave rise to lignin-yielding derivatives with no measurable radioactivity. In view of the fact that buckwheat can metabolize *p*-hydroxycinnamic acid (12), the data in Table I suggest that the metabolic difference in this species concerns the formation of this compound from *p*-hydroxyphenyllactic acid. It may be that no enzyme exists in this species capable of mediating the reactions amounting to a dehydration in the 2,3-position when the ring is hydroxylated. If the same enzyme is concerned in the dehydration of both hydroxylated and non-hydroxylated substrate, a lower degree of specificity in the grasses is indicated. At present this can only be speculation.

In experiment 4, Table I, phenylalanine was not extensively converted to syringyl lignin by buckwheat, in contrast to experiment 3, in which guaiacyl and syringyl lignin were formed about equally well. These and other (unpublished) results lend support to the belief that at certain times one lignin monomer may be formed very slowly while another is being formed rapidly. This point deserves further study.

It should be noted, too, that in buckwheat no carbon-14 was found in *p*-hydroxybenzaldehyde after administration of radioactive *p*-hydroxyphenyllactic acid. If the latter is formed from *p*-hydroxyphenylpyruvic acid by a reversible reaction, the equilibrium must be strongly shifted in the direction of the hydroxy acid. Otherwise, the conversion of *p*-hydroxyphenyllactic acid to tyrosine and the appearance of radioactivity in *p*-hydroxybenzaldehyde would have been expected.

An additional test of the existence of a metabolic block in species other than grasses should be available in the ability of plants to form *p*-hydroxycinnamic and ferulic acids from tyrosine. In grasses the degree of conversion of tyrosine to these widely occurring acids should be much greater than in non-grasses. Table II shows the dilution of carbon-14 during the conversion of randomly labelled L-phenylalanine and L-tyrosine to *p*-hydroxycinnamic and ferulic acids in wheat. The analogous data of McCalla (7) for salvia are included for comparative purposes. Whereas in wheat *p*-hydroxycinnamic acid is formed 1.6 times and ferulic acid 3.5 times more efficiently from phenylalanine than from tyrosine, the respective figures for salvia are 13 and 75. These results also point to the existence of a more direct route (presumably via *p*-hydroxyphenyllactic acid) from tyrosine to these acids in wheat than is available to salvia.

TABLE II

Conversion of phenylalanine and tyrosine to *p*-hydroxycinnamic and ferulic acids in wheat and salvia

	Compound administered			Specific activity of isolated compound, μc/mM		Dilutions (corr. for plant wt.)*	
Species	Name	Specific activity, μc/mM	Dose, μM/g dry wt.	*p*-Hydroxycinnamic acid	Ferulic acid	*p*-Hydroxycinnamic acid	Ferulic acid
Wheat	L-Phenylalanine	1955	1.4	8.99	7.33	218	267
	L-Tyrosine	1580	1.3	4.56	1.71	347	925
Salvia†	L-Phenylalanine	3240	0.92	8.0	2.56	400	1300
	L-Tyrosine	3780	1.6	0.82	0.045	5300	98,000

*Observed dilution equals value in column 3 divided by that in columns 5 or 6.
†Data for salvia are those of McCalla (7).

It is now possible to propose a partial scheme covering the reactions from prearomatic compounds to lignin which is consistent with the facts at hand. This is presented in Fig. 1. In this scheme it has been necessary to assume the participation of phenylpyruvic acid as an intermediate, as it is only on this assumption that it appears possible to explain the very efficient utilization of both phenylalanine and shikimic acid (13) in lignification. Feeding of labelled phenylpyruvic acid has shown this compound to be utilized less efficiently than phenylalanine, but this may be explainable on the basis of instability of the keto acid, or the necessity of its conversion to some "active" form such as phosphoenolphenylpyruvic acid.

It has already been demonstrated that shikimic acid is an efficient precursor of lignin (13), and that there is no significant randomization during this conversion (14); this leads to the inference that, as in the case of microorganisms (15), prephenic acid is an intermediate between shikimic acid and

fully aromatized compounds. Ghosh (16) has presented evidence that in *Escherichia coli* prephenic acid is converted to *p*-hydroxyphenyllactic acid without passing through phenylpyruvic acid, and Schwink and Adams (17) have recently reported that *p*-hydroxyphenylpyruvic acid is an intermediate in this conversion. From this last compound tyrosine has now been shown to arise in buckwheat, probably by transamination. An alternative origin for *p*-hydroxyphenylpyruvic acid would be hydroxylation of the aromatic ring of phenylpyruvic acid. Again on the assumption that phenylpyruvic acid is readily interconvertible with phenylalanine, this amino acid should serve as a good tyrosine precursor if this alternative route is predominant. In salvia, phenylalanine is converted to tyrosine to some extent (7), and this is consistent with the insertion of the ring hydroxyl at the oxidation state of phenylpyruvic acid. The relative importance of this less direct route is still unassessed.

Prephenic acid is tentatively taken in the present scheme as the branching point for two routes to *p*-hydroxycinnamic acid, a compound which we believe to be a key intermediate in lignification. In the first pathway phenylpyruvic acid is reduced to cinnamic acid through phenyllactic acid (18) and the ring is hydroxylated at the cinnamic acid stage. In view of the efficient utilization of phenylalanine for lignin biosynthesis in several widely differing species it is probable that this pathway is widely if not universally used by

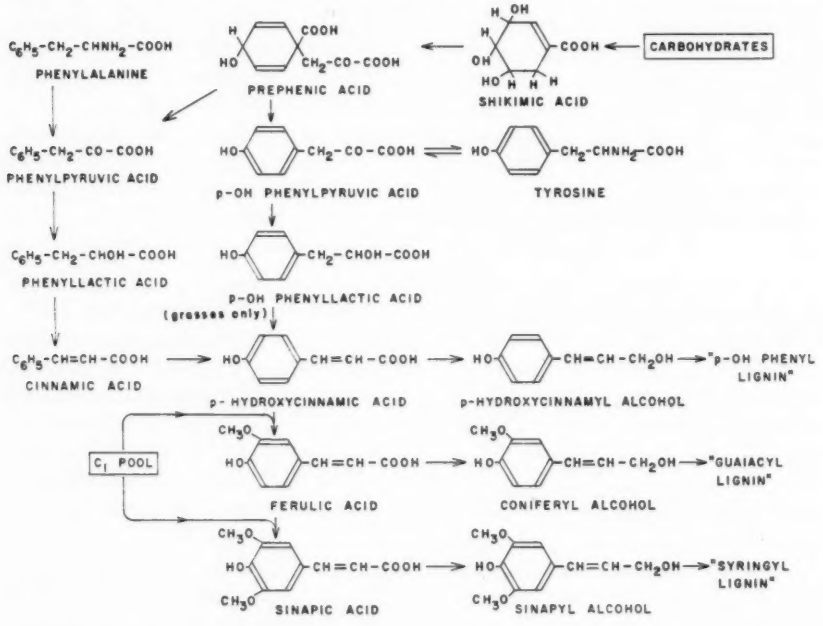

FIG. 1. Proposed metabolic interconversions of phenylpropanoid compounds in lignification.

plants. In the second pathway the *p*-hydroxyphenyllactic acid formed (via *p*-hydroxyphenylpyruvic acid) from prephenic acid is dehydrated to *p*-hydroxycinnamic acid. It is this route which present evidence indicates to be confined to the grasses.

p-Hydroxycinnamic acid, however formed, would be subject to competing reactions leading both to the reduction of the carboxyl group and to additional ring hydroxylation. The former would lead to *p*-hydroxycinnamyl alcohol, the latter to ferulic and sinapic acids. The good conversion of caffeic acid demonstrated previously (2) makes it very likely that it is an intermediate in the latter reaction with the methyl groups arising through transmethylation (19) and ultimately from some one-carbon intermediate of photosynthesis (20). Reduction of the methoxylated acids in turn would yield, respectively, coniferyl and sinapyl alcohols, and polymerization or copolymerization of the three alcohols would be the final step (4) in lignification.

It should be noted that this scheme takes no account of, but does not rule out, glycosidation, which is believed to play a role in the transport of the relatively insoluble alcohols to the site of lignification (21), or such reactions as sulphydryl ester formation, known to be an essential aspect of the metabolism of other organic acids. Also, the fact that most reactions are not shown as being reversible is intended to indicate only that reversibility has not been conclusively demonstrated. Indeed, it seems likely that many of them are reversible, with the equilibrium under the usual conditions being displaced in the direction of synthesis.

The authors are under no illusions that Fig. 1 shows the only paths through the jungle of phenylpropane derivatives. The interconversions shown are perhaps the minimum necessary to explain the observed facts, but in view of the evident facility with which plants can alter the oxidation state both of the side chain and the ring, additional pathways are clearly possible. The precise role of phenylalanine in lignification, for example, is still far from being elaborated. Most other lignin precursors thus far investigated, including several which more closely resemble in structure the assumed lignin monomers, are used less efficiently than phenylalanine. Shikimic acid, too, is more efficiently used than its position rather remote from lignin would lead one to expect. These anomalies may point to as yet unsuspected alternative routes, or may be an indication that these two compounds more readily undergo the "activation" necessary for their further metabolism in the plant.

The results of the experiments with tyrosine and *p*-hydroxyphenylpyruvic acid, as well as those dealing with the utilization of enantiomorphs (18) by different species, are evidence of the value of comparative studies in biogenetic investigations, and serve to underline the dangers inherent in proposing general reaction pathways based on work with a single species.

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NOTE ADDED IN PROOF: Since the submission of this paper, G. Billek (Symposium on biochemistry of wood. IVth International Congress of Biochemistry, Vienna, 1958.) has reported that spruce, too, is unable to convert *p*-hydroxyphenylpyruvic acid to lignin. This provides additional support for the idea that the ability to utilize this compound is very restricted in the plant kingdom.

COMPARISON OF CARDIOTOXIC ACTIONS OF CERTAIN SYMPATHOMIMETIC AMINES[1]

CLIFFORD I. CHAPPEL, GEORGE RONA, TIBOR BALAZS, AND ROGER GAUDRY

Abstract

Rats were injected subcutaneously on 2 consecutive days with various doses of four sympathomimetic amines. The doses chosen were the median lethal dose or fractions thereof. The animals were sacrificed at 48 hours and myocardial lesions were graded in the gross and after histological sectioning. A synthetic adrenergic agent, isoproterenol, was found to cause severe infarct-like myocardial necrosis. The lesions which were seen after isoproterenol treatment were more severe than those produced by *l*-epinephrine, *l*-arterenol, or ephedrine. This was the case whether the severity of the lesions was expressed in terms of single dose lethality of the compound or in terms of absolute amount. It was concluded that the myocardial lesions produced by these drugs are related to their cardiac stimulant properties.

Introduction

Soon after the discovery of epinephrine Josué (1) showed that this agent could produce aortic lesions in the rabbit. In his second paper (2) he also mentions the presence of myocardial hypertrophy in these animals. Fleisher and Loeb (3) later showed that epinephrine or epinephrine combined with sparteine would cause myocarditis in this species. Anitschkow (4), who reviewed this field in 1913, confirmed these earlier findings and described in detail the myocardial lesions. Veith (5) observed disseminated myocardial necrosis in the cat following infusions of epinephrine. The lesions which were localized principally in the anterior papillary muscle were compared to similar lesions which are seen in shock (6, 7). Veith attributed the necrosis in either case to myocardial hypoxia. Recently Waters and de Suto-Nagy (8) found that epinephrine infusions in the dog caused necrosis in the aorta, and in the coronary and pulmonary arteries with interstitial edema and focal necrosis in the myocardium. Vishnevskaya (9) has reported myocardial lesions in rats after single injections of 0.5 mg of epinephrine. According to this author the lesions were primarily inflammatory and were termed "myocarditis". King (10) was able to produce infarct-like myocardial lesions by the intravenous infusion of epinephrine or arterenol to the dog. Similar lesions were produced with these compounds by Nahas *et al.* (11) in heart–lung preparations.

Recent reports from this laboratory (12, 13) have shown that 1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride (isoproterenol) administered subcutaneously to the rat caused infarct-like myocardial necrosis. The severity of the lesions was directly proportional to the dosage, so that lesions of predictable severity could be produced. These reports were the first disclosure of severe morphological changes in the heart after the

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Contribution from Research Laboratories, Ayerst, McKenna & Harrison Ltd., Montreal, Canada.

administration of a synthetic adrenergic agent. The lesions were more uniform and appeared to have greater severity than those reported for adrenaline or arterenol. Because of the possible therapeutic implications of these findings we have studied in detail a number of sympathomimetic amines. This report compares the myocardial lesions produced by isoproterenol, *l*-epinephrine, *l*-arterenol, and ephedrine in the rat.

Experimental Procedure

The rats used in this study were Wistar strain fed a commercial rat diet and housed in an air-conditioned room at a temperature of 75° F. As a basis for the choice of doses used in subsequent tests a single dose acute toxicity test was performed on each drug to determine the median lethal dose (LD_{50}). Each compound was administered subcutaneously in aqueous solution at four dose levels to a group of 10 animals. The mortality rate in the groups at 24 hours was recorded, and used to calculate the LD_{50} by the method of Litchfield and Wilcoxon (14). In the studies of cardiotoxic properties each compound was administered subcutaneously to five male and five female rats on 2 consecutive days. The first group was treated with a dose corresponding to the single dose LD_{50}; thereafter each consecutive dose was one half the preceding dose. Both epinephrine and arterenol were administered as the bitartrates of the *l*-isomer, and isoproterenol and ephedrine as the hydrochlorides of the racemic mixture. The results in each case are expressed in terms of the base. A control group received distilled water. Forty-eight hours after the first injection the rats were killed and autopsied. The hearts were removed, weighed, and the gross lesions were graded according to the following system: Grade 0, no lesions. Grade 1, mottling of the apex and distal parts of the left ventricle caused by intermingled pale- and dark-red streaks. Grade 2, well-demarcated necrotic areas limited to the apex. Grade 3, large infarct-like necrosis involving at least one third of the left ventricle and extending to adjacent areas of the interventricular septum and right ventricle. Grade 4, large infarct-like necrosis involving more than half of the left ventricle, the interventricular septum, and extending to the distal portion of the right ventricle.

After fixation in Bouin's fluid frontal sections of the heart, which included both atria, ventricles, and interventricular septum, were embedded in paraffin. Sections cut at 5 μ were stained with hematoxylin–eosin, Cason's trichrome (15), and also studied with the periodic acid Schiff reaction (P.A.S. (16)). Frozen sections of the heart were stained with Sudan IV. Microscopically the hearts were graded as follows: Grade 0, no lesions. Grade 1, focal lesions of the subendocardial portion of the apex and/or the papillary muscle composed of fibroblastic swelling or proliferation and accumulation of histiocytes. Grade 2, focal lesions extending over wider areas of the left ventricle with right ventricular involvement. The lesions included also edema, mottled staining, fragmentation, and segmentation of muscle fibers. Grade 3, confluent lesions of the apex and papillary muscles with focal lesions involving

other areas of the ventricles and the atria. The lesions included vacuolar and fatty degeneration, granular disintegration, and hyaline necrosis of the muscle fibers. Marked capillary dilatation was present with hemorrhages, pronounced mononuclear cell, and leukocytic infiltration. Extensive edema, occasionally with a mucoid component, caused sequestration of muscle fibers. Grade 4, confluent lesions throughout the heart including infarct-like massive necrosis with occasionally acute aneurysm or mural thrombi. The infarcts were usually apical but also occurred in the papillary muscles or the right ventricle. The histologic lesions were similar in character to those in grade 3. Histological sections were also made of other organs in which gross pathology was observed and in two additional animals from each group sections were made of the brain, pituitary, lung, liver, kidney, adrenal, and pancreas.

Results

Mortality and Pathological Findings

The median lethal dose of isoproterenol after a single subcutaneous dose was found to be 581 mg/kg. One hundred and eighty animals were treated with isoproterenol in groups of 10 at 18 dose levels which were fractions of the LD_{50}. The average weight of the males was 258 ± 10 g and the females 200 ± 4 g. When the LD_{50} dose, 581 mg/kg, was administered to a group of rats on 2 consecutive days the mortality was 80%. Doses of 290.5, 145.2, 72.6 mg/kg caused the death of 90, 50, and 10% of the animals, respectively, during the experimental period. Sporadic deaths were observed at lower doses, one at 18.1 and two at 1.1 mg/kg. These deaths occurred in animals with particularly severe cardiac lesions. At autopsy, lesions were found outside the heart at dose levels of 145.2 mg/kg and more. These consisted of centrolobular liver necrosis and hemorrhage in 40 to 50% and cortical ischemia or necrosis of the kidney in 10 to 40% of the rats, with evidence of severe passive congestion. At doses between 72.6 and 1.1 mg/kg no necrotic changes were observed outside the heart, but pulmonary edema, congestion of the liver, spleen, kidney, gastrointestinal tract, and leptomeninges with cerebral swelling were still observed in 30 to 100% of the animals at these doses. At autopsy the hearts of most of the animals receiving isoproterenol were obviously enlarged, and were found to be heavier than the controls. The difference in weight compared with the controls was significant at the 99% level of confidence for all groups receiving doses of 4.5 mg/kg or more.

The LD_{50} of *l*-epinephrine after a single dose was 6.1 mg/kg. Groups of 10 animals, in which the males weighed an average of 179 ± 7 g and the females weighed 210 ± 29 g, were given the LD_{50} dose or a fraction of this dose on 2 consecutive days. At the 6.1 mg/kg four animals died from the group of 10 and at 3.1 mg/kg two animals died. The animals treated at these doses had lung edema with hemorrhage, centrolobular liver necrosis, and cortical renal necrosis. A significant increase in heart weight occurred in the animals receiving 0.74 mg/kg or more.

The LD_{50} of *l*-arterenol after a single dose was 8.6 mg/kg. Six groups of 10 rats were treated on 2 consecutive days with the LD_{50} or a fraction thereof.

Both males and females weighed an average of 174 ± 2 g. Seven of 10 animals died at 8.6 mg/kg, 4 at 4.3 mg/kg, and 2 at 2.1 mg/kg. The autopsy findings were similar in character and severity to those described for *l*-epinephrine. The hearts were enlarged significantly in animals treated with 1.1 mg/kg or more.

The LD_{50} of ephedrine after a single dose was 531 mg/kg. This dose and doses of 266, 133, and 66.5 mg/kg were administered to groups of rats on 2 consecutive days. The average weight of the males was 210 ± 4 g and that of the females 180 ± 4 g. Five rats died from the group treated with 531 mg/kg and one from the 266 mg/kg dose. At autopsy no changes were seen in any of the groups. The hearts were not significantly enlarged.

Comparison of Cardiac Lesions

The average grades of the gross heart lesions of each group have been plotted in Fig. 1. The mean severity of the lesions produced by each drug is plotted against the respective median lethal dose or fraction thereof. Ephedrine produced only occasional indistinct lesions at the highest doses. *l*-Epinephrine and *l*-arterenol are equally active in producing cardiac lesions. However, both compounds must be administered in comparatively high doses in terms of the LD_{50} in order to cause lesions demonstrable in the gross. At 0.37 mg/kg *l*-epinephrine produced no gross lesions while 0.55 mg/kg caused indistinct cardiac lesions in only 1 animal out of 10. Isoproterenol produced lesions of almost maximal severity at a dose of 72.6 mg/kg. Nine of the 10 animals in this group had infarct-like myocardial necrosis involving the apex, the lower part of the left ventricle, the interventricular septum, and occasionally the

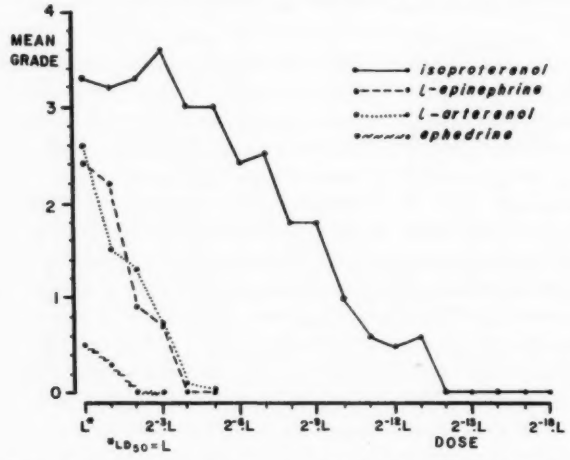

FIG. 1. Comparison in the gross of myocardial lesions produced by four sympathomimetic amines. Doses on the abscissa are fractions of the respective median lethal doses which were administered on 2 consecutive days. Each point is the average of the grading of 10 animals.

right ventricle (Fig. 2). The mean grade of the lesions of rats receiving higher doses is somewhat less because 40 to 50% of these animals died after only one dose of isoproterenol and too soon for the development of lesions of maximal severity. As the dose level of isoproterenol was reduced the lesions found in the rats were less severe; however, a dose of 1.1 mg/kg produced lesions of an average grade of 1.8 in 100% of the animals. The lowest dose which resulted in lesions which could be recognized in the gross was 0.068 mg/kg. It is obvious from the data in Fig. 1 that isoproterenol is more potent than the other compounds in causing gross cardiac lesions when these are expressed in terms of the lethality of the compound. Although isoproterenol is almost 100 times less toxic in the rat than *l*-epinephrine or *l*-arterenol, the same is true when the doses are expressed in absolute terms. The amounts of each compound which are required to produce gross lesions of equal severity are shown in Table I. When isoproterenol is compared with *l*-epinephrine and *l*-arterenol at grade 1.5, 1.0, 0.5 it may be seen that the latter two compounds require at least two to six times the dose of isoproterenol to cause myocardial lesions of similar grade. At grade 2.0 this difference between *l*-epinephrine and isoproterenol is not apparent, in fact it may be seen that there is an inverse relationship between the grade and the difference between amounts of isoproterenol and *l*-epinephrine or *l*-arterenol that are required to produce similar lesions.

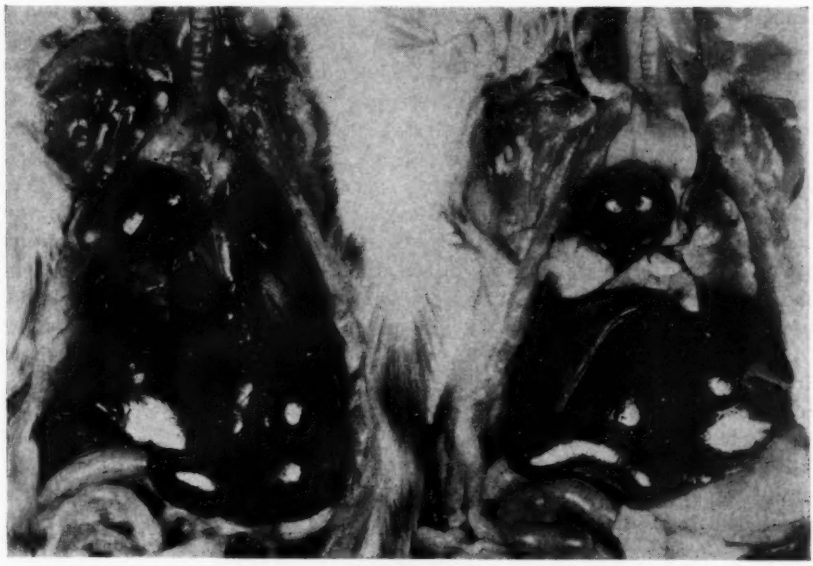

FIG. 2. On the left, infarct-like myocardial necrosis, edema and congestion of the lungs, and passive congestion of the abdominal viscera in a rat treated with 72.6 mg/kg of isoproterenol subcutaneously on 2 consecutive days. Animal on the right is normal control.

TABLE I

Comparison of four sympathomimetics, absolute amounts which would be required to produce myocardial lesions of equal severity

Compound	Gross grade				Microscopic grade			
	2.0	1.5	1.0	0.5	2.0	1.5	1.0	0.5
Isoproterenol	2.905*	0.959	0.568	0.116	0.0354	0.0186	0.0139	0.0087
l-Epinephrine	2.745	2.135	1.525	0.552	1.830	1.343	0.763	0.429
l-Arterenol	6.435	4.290	1.630	0.774	1.030	0.772	0.536	0.257
Ephedrine	–	–	–	531.0	–	–	238.95	159.30

*Amount of compound in mg/kg.

Ephedrine caused microscopic changes of the heart in 50 to 70% of the animals treated on 2 consecutive days with 531 or 266 mg/kg. The lesions were focal and composed of capillary dilatation, interstitial edema, histiocytic infiltration, fibroblastic swelling, and proliferation. The regressive changes of the muscle fibers were mild or absent; only a mottled staining of the involved muscle fibers could be observed. At a dose level of 133 mg/kg 2 out of 10 animals had mild lesions and at 66.5 mg/kg no microscopic changes were seen. *l*-Epinephrine administered at dose levels of 1.5 mg/kg or greater caused focal myocardial necrosis and prominent reactive inflammation. In 10 to 30% the necroses were confluent with hemorrhagic perimeters and mural thrombi were seen. Only mild inflammatory changes were seen at 0.37 mg/kg or less. The histological lesions produced by *l*-arterenol (Fig. 3) were similar in characteristics and severity to those obtained by *l*-epinephrine. Confluent necroses occurred in 10 to 40% of cases at 1.1 mg/kg or more. Isoproterenol produced infarct-like massive necrosis from 581 mg/kg to 0.28 mg/kg (Fig. 4). The necrosis often involved the whole thickness of the left ventricle causing acute aneurysm and mural thrombus formation. At 0.068 mg/kg all the animals had microscopic lesions. The most characteristic change of the muscle fibers was hyaline necrosis. This was present in 70 to 100% of the hearts of the animals receiving doses of 4.5 mg/kg or more and was the primary component of the massive infarct-like necrosis. The results of the grading following microscopic examination, in general, parallel those of the gross observations. Isoproterenol was found to be the strongest cardiotoxic agent as indicated in Fig. 5 where mean severity is plotted in terms of the LD_{50}. The same is true when the drugs are compared in terms of absolute doses, as is shown in Table I. The differences between isoproterenol and *l*-epinephrine or *l*-arterenol are more pronounced when the lesions are graded histologically than in the gross. At grades 2.0, 1.5, 1.0, or 0.5 isoproterenol is 29 to 72 times more potent in producing myocardial lesions of equal severity than *l*-epinephrine or *l*-arterenol. None of the 10 control rats had any gross or microscopic heart lesion.

Discussion

It has been known for many years that epinephrine (4, 5, 8) and, more recently, that arterenol (10, 11) could produce organic heart changes when administered by intravenous infusion to a number of species. The studies

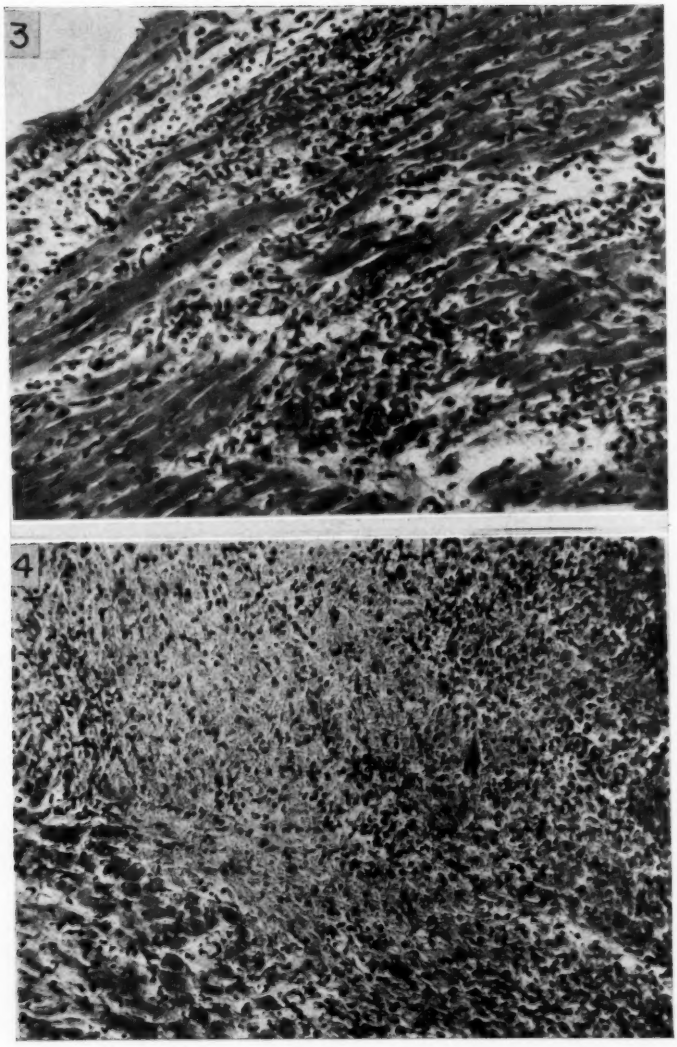

FIG. 3. Areas of granular disintegration, edema, and cellular infiltration in the left ventricle of a rat treated with 2.1 mg/kg of *l*-arterenol subcutaneously on 2 consecutive days. Lesion is primarily inflammatory. Haematoxylin–eosin, ×120.

FIG. 4. Area of hyaline necrosis with histiocytic reaction and fibroblastic proliferation. Lesion present in the left ventricle of a rat treated with 72.6 mg/kg isoproterenol subcutaneously on 2 consecutive days. Haematoxylin–eosin, ×120.

reported here demonstrate this property in a synthetic sympathomimetic compound, isoproterenol, administered subcutaneously and show that this compound is more potent in this respect than *l*-epinephrine or *l*-arterenol when compared on the basis of single dose lethality or absolute amounts.

Though the cardiac necrosis produced by isoproterenol corresponds in gross and histological characteristics to that of ischemic necrosis of man and experimental animals, no changes in the coronary arteries were observed which suggest a disturbance of coronary circulation. Since Denison *et al.* (17) have shown that this compound causes dilatation of the coronary vessels, vascular spasm or occlusion would not appear to be implicated in the etiology of these lesions.

As shown by Figs. 1 and 5 there is a good correlation between the severity of the lesions produced by isoproterenol and the dose tested. This finding plus the demonstration that doses as low as 1/512 of the LD_{50} produced infarct-like cardiac necrosis suggested that the cardiotoxicity of this drug is related to its cardiovascular effects rather than being a direct toxic effect exerted on the heart. The pharmacological actions of isoproterenol on the cardiovascular system compared with those of epinephrine or arterenol would appear to support such a concept. Acute coronary insufficiency arises when the balance between oxygen need and blood supply of the myocardium is disturbed. The immediate result will be hypoxemia of the myocardium followed in severe cases by necrosis. Epinephrine is believed to cause myocardial lesions by cardiac stimulation which increases the oxygen requirements of the heart muscle so that in spite of coronary vasodilatation a relative insufficiency of oxygen is produced (18). It has been shown by Lands and Howard (19) that isoproterenol is 16 times more active in inotropic effect and 36 times

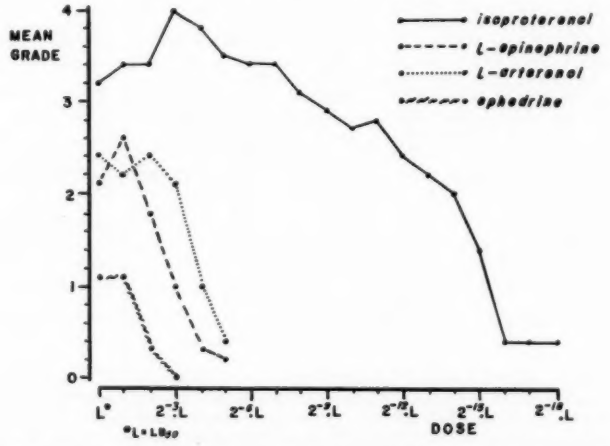

FIG. 5. Microscopic grading of myocardial lesions produced by four sympathomimetic amines. Doses on the abscissa are fractions of the respective median lethal doses which were administered on 2 consecutive days. Each point is the average of the grading of 10 animals.

more active in chronotropic effect than epinephrine when tested in the isolated perfused frog heart. It also has greater activity on rhythmic properties of the human heart than epinephrine (20), and isoproterenol-induced arrhythmias require greater doses of an adrenergic blocking drug for a protective effect than do those induced by epinephrine (21). Since isoproterenol is a stronger cardiac stimulant than the other amines, it would be expected to induce a correspondingly greater need for oxygen by the heart muscle; at the same time the depressor effect of this compound on the systemic circulation would prejudice the oxygen supply to some extent. The stimulant effect of isoproterenol on the heart and its depressor properties on systemic circulation, we believe, account for the myocardial necrosis which is produced.

The organic lesions which we have found in the rat after isoproterenol treatment are not mentioned in the toxicity studies of Dertinger *et al.* (22) and Lands *et al.* (23). These authors found no significant gross or histopathological abnormalities after the administration of 100 mg/kg of this drug subcutaneously for 15 days. It is unlikely that these differences can be explained on the basis of the strain of rat used, since we have found equally severe lesions in two strains of albino rats and one strain of hooded rats. A possible explanation for this apparent contradiction may lie in the difference in body weight of the animals used. Whereas our rats had a mean weight of 229 g, those used by Dertinger weighed from 75 to 136 g. In recent studies we have found a clear correlation between the severity of the lesions and the body weight of rats treated with isoproterenol (24). Animals which weigh less than 100 g all develop lesions, but they are less severe than those observed in older heavier rats.

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ALCOHOL LEVELS IN BODY FLUIDS AFTER INGESTION OF DISTILLED SPIRITS[1]

B. B. COLDWELL[2] AND H. WARD SMITH[3]

Abstract

Known volumes of 70-proof distilled spirits were fed to 68 volunteers, making a total of 141 separate doses. The amount of alcohol appearing in the venous blood, saliva, and urine at various time intervals after ingestion was determined by a modified Widmark method, and in the breath by the Breathalyzer. The relationship between time after ingestion, venous blood alcohol concentration (VBA), and Breathalyzer reading (BR) was as follows:

(1) between 0.5 and 2.5 hours after ingestion, VBA = (BR + 0.048) ± 0.124 mg/ml;

(2) between 30 to 40 minutes after ingestion, VBA = (BR − 0.071) ± 0.065 mg/ml;

(3) between 2 to 2.5 hours after ingestion, VBA = (BR + 0.102) ± 0.117 mg/ml. The weighted average ratios of saliva and urine alcohols to venous blood alcohol were 1.12:1 and 1.24:1, respectively. The standard error of estimating the venous blood alcohol indirectly from the saliva alcohol was ±0.075 mg/ml, and from urine alcohol ±0.081 mg/ml when the samples were obtained from 0.5 to 2.5 and from 0.75 to 2.5 hours after drinking, respectively. Over the range of concentrations studied alcohol disappeared from the venous blood at the rate of 0.13 ±0.05 mg/ml/hr and the quantity eliminated from the whole body, per 100 lb of body weight per hour, approximated 0.4 fl. oz of 70-proof distilled spirits.

Introduction

In medicolegal investigations, blood is widely accepted as the standard body material for the analysis of alcohol in living persons (1). On this continent the blood sample is usually taken from the arm vein. However, obtaining a sample of blood may present a number of immediate practical problems (1, 2) and these have prompted various investigators to suggest the use of breath (3, 4, 5, 6, 7), saliva (3, 8), and urine (9, 10, 11).

The indirect measurement of the concentration of alcohol in venous blood by using breath is based on the relation that 2100 parts of alveolar air (deep lung breath) contains the same weight of alcohol as 1 part of blood (12). The estimation of blood alcohol from the quantity in saliva or urine is based on the fact that various workers have reported fairly constant ratios between the blood alcohol level and the alcohol level in each of these fluids (9, 13, 14). Criticism of these indirect methods has been mainly on physiological grounds (15).

The experiments reported here were carried out in connection with an investigation into the effects of alcohol on the driving performance of experienced car drivers (16), with the object of obtaining additional data on the relation between the alcohol content of an individual's venous blood, breath, saliva, and urine.

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Contribution from the Crime Detection Laboratories, Royal Canadian Mounted Police, Ottawa, and The Attorney General's Laboratory, Province of Ontario, Toronto. Presented in part at the Forty-first Annual Conference of the Chemical Institute of Canada at Toronto, Ontario, May, 1958.

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Experimental

The 68 male volunteers ranged in age from 24 to 58 years and in weight from 125 to 224 lb. The liquor consumed (70-proof distilled spirits, approx. 40% alcohol by volume) by each volunteer was divided into three equal portions, diluted to taste with the preferred mixer (water, Coca-Cola, ginger ale, Tonic Water, soda water), and 20 minutes allowed to drink each portion. For example, one subject consumed 3 fl. oz of Scotch whisky, diluted to taste with water, between 1.00 and 1.20 p.m., another 3 fl. oz, similarly diluted, between 1.20 and 1.40 p.m., and a final 3 fl. oz between 1.40 and 2.00 p.m. Potato chips were available during drinking. Except for a few exploratory experiments, the amount of liquor given to each volunteer depended initially on his body weight (2 fl. oz per 50 lb body wt.) and subsequently on his driving performance (16). Eighteen subjects drank on one occasion, 38 on two, 1 on three, and 11 on four separate days making a total of 141 separate tests. All subjects began drinking between 1 and 2 hours after eating a meal.

Breath, blood, saliva, and urine samples were taken for alcohol analysis at various times after the end of drinking.[4] Breath samples were analyzed with the Breathalyzer[5] which converts the breath alcohol content directly into the percentage of alcohol in the arterial blood. Venous blood samples were taken from the arm by an experienced hospital technician. The arm was cleaned with liquid soap before the sample was taken. Saliva samples were obtained by having the subject chew a small cube of paraffin wax and expectorate into a small bottle. Urine samples were collected by having the subject void into a clean container. A portion of each sample of body fluid, when obtained, was immediately transferred to a 10-ml test tube containing an anticoagulant preservative (75 mg sodium fluoride and 50 mg sodium citrate). The tube was stoppered and the contents were mixed. Analysis, by Smith's modified Widmark procedure (17) was usually completed on the day of the experiment; if any delay occurred the samples were refrigerated until analyzed, which was always within 24 hours of the time of taking the sample.

Results and Discussion

Blood and Breath

In Fig. 1, the alcohol levels determined directly in venous blood samples are plotted against the estimated Breathalyzer readings for the times the blood samples were taken from the subjects. These estimated readings were obtained by interpolation from the values recorded on breath samples taken at known times. No estimates were made unless breath samples were taken within 5 minutes of, or both before and after the blood sample was obtained. In eight comparisons the amount of alcohol in the venous blood was estimated

[4]The complete analytical results together with the times of taking the samples, the kind and quantity of liquor consumed, and the age and weight of each volunteer are omitted for reasons of space. Readers will find this information in reference 16 or it is available on request from one of the authors (B.B.C.).

[5]The Breathalyzer was invented by R. F. Borkenstein, Chairman, Department of Police Administration, Indiana University, Bloomington, Indiana, and is distributed by the Stephenson Corp., Red Bank, New Jersey, U.S.A.

for the time of taking the breath sample. In about 34% of the 253 comparisons the breath samples were taken within five minutes of the times the blood samples were obtained. In just over one half of the comparisons the elapsed times between the two breath samples used in the interpolations ranged between 30 and 60 minutes; the time exceeded 60 minutes in only 3% of the comparisons.

From Fig. 1, it is evident that in the majority of cases Breathalyzer readings gave a lower estimate of the blood alcohol concentrations than direct analysis of the blood samples. The average of the differences between the values obtained by the two methods of analysis is -0.048 mg/ml with a standard error of ± 0.008. That is, the interpolated Breathalyzer values, on the average, underestimated the venous blood alcohol levels by 0.048 mg/ml. When this correction was applied, the interpolated Breathalyzer reading estimated the venous blood alcohol concentration with an accuracy of ± 0.124 mg/ml. It is further indicated by Fig. 1 that the magnitude of the differences between the results of the two methods of analysis bears no relation to the actual concentration of alcohol in the venous blood.

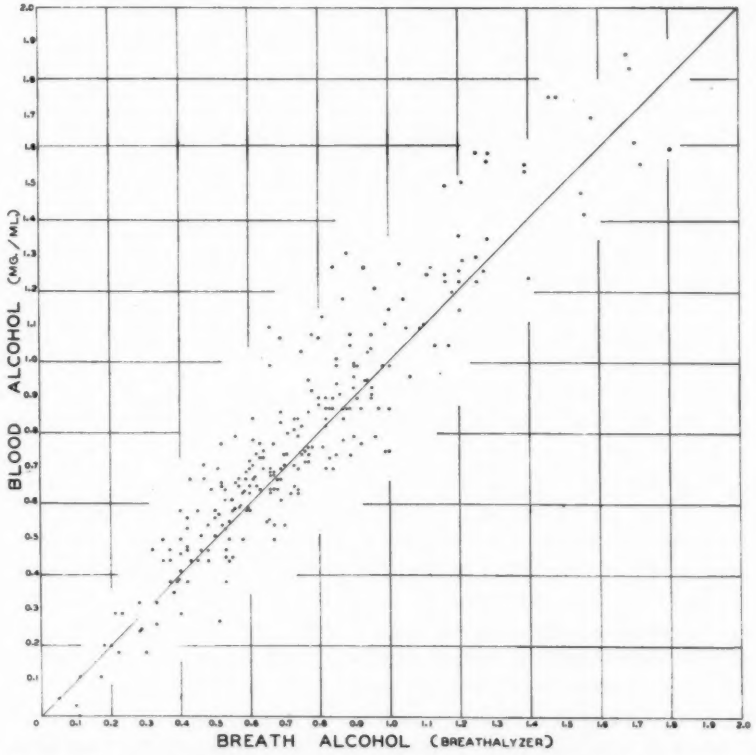

FIG. 1. Relationship between the amounts of alcohol found present in venous blood on direct analysis of blood samples and indirectly by analysis of breath samples.

In 87 of the blood–breath comparisons, the breath samples were taken within five minutes of the time the blood samples were drawn from the arm. In 10 of these, the samples were obtained within 30 to 40 minutes of the time the subject stopped drinking; in 9 of the 10 the Breathalyzer reading was higher than obtained by direct analysis of the blood sample, the mean difference being $+0.071 \pm 0.065$ mg/ml. Student's t test for paired differences (18) established that the probability is about 0.01 (i.e. in about 1 out of 100 tests) that this consistent difference would occur solely by chance. Therefore, it would appear from these results that during the first 40 minutes after the end of drinking, the Breathalyzer gives a higher estimate of the alcohol concentration in the venous blood than is obtained by direct analysis. This result was expected on physiological grounds as discussed below. Of the remaining 77 comparisons, in 16 the Breathalyzer reading was higher than, in 1 it was equal to, and in 60 it was lower than the alcohol levels found on direct analysis of the blood samples. The average difference calculated for these 77 comparisons was -0.102 ± 0.117 mg/ml. Most of these blood and breath samples were obtained between 2 and 2.5 hours after the end of drinking. The probability is very slight (<0.001) that pairs of samples selected at random would exhibit these differences by chance. Four out of every five Breathalyzer readings taken 2 hours or more after the end of drinking will give a lower estimate of the alcohol level in venous blood than the estimate determined by direct analysis. Further investigation is necessary to establish whether this apparent relationship between time elapsed since drinking, Breathalyzer reading, and venous blood alcohol level has any significance for the individual person. It has been noted previously (16), that no systematic change was evident in the magnitude of the difference between the alcohol concentrations in breath and blood samples taken from one person at various intervals after drinking. However, these calculations included some interpolated values which might have obscured differences between the two methods of analysis. Such a relationship would be expected on physiological grounds. The amount of alcohol which enters the lung air is proportional to the concentration of alcohol in the blood which leaves the lungs to enter the left side of the heart (4, 19), from where it is pumped through the arteries to all parts of the body. The venous blood in the arm vein has just passed through the capillaries of the hand and arm and in its passage has lost alcohol to the tissues. As time passes the rate of loss into the tissues becomes slower and the level of alcohol in the venous blood increases and becomes nearly equal to that in the arterial blood. In most individuals this equilibrium is established within 45 to 60 minutes after the end of drinking (20). The level of alcohol in the breath, which reflects that in the arterial blood, therefore should be higher than the level in the venous blood for a short interval immediately following ingestion of alcohol.

The fact that the Breathalyzer reading in the majority of tests was lower than the result obtained by direct analysis of the corresponding venous blood sample, has a logical explanation. The Breathalyzer is calibrated on the

assumption that the breath being analyzed is pure alveolar air, that is air from deep in the lungs (7). In practice, the breath sample obtained is frequently a mixture of alveolar and recently inhaled air; the alcohol content of such a breath sample will be less than in pure alveolar air. Further, the Breathalyzer is so constructed that any mechanical failure must result in a low, rather than a high reading. Hence, the Breathalyzer reading tends to underestimate the level of alcohol in the blood reaching the brain at any instant following ingestion.

Blood and Saliva

The concentrations of alcohol in 244 samples of saliva obtained from the volunteers in these experiments are compared with the corresponding venous blood samples in Fig. 2. In 29 of these comparisons, the saliva alcohol concentration was estimated, for the time the blood sample was taken, by interpolating between the two appropriate samples. In most of the remaining comparisons (210 out of 215), the saliva samples were obtained within 10 minutes of the corresponding blood samples and no attempt was made to correct for this time difference. The saliva alcohols plotted in Fig. 2 were divided by 1.12, the weighted mean ratio of blood to saliva alcohol found in these experiments. This ratio was calculated by dividing the sum of the venous blood alcohol concentrations into the sum of the corresponding saliva alcohol concentrations.

It is evident from Fig. 2 that there is a high correlation between the concentration of alcohol in saliva and in venous blood. Also, there is no suggestion that the magnitude of differences between corresponding venous blood and saliva alcohols is related to the blood alcohol level. Investigation revealed no correlation between these differences and time from the end of drinking, during the period from 0.5 to 2.5 hours following ingestion. The saliva alcohol, divided by 1.12, estimated the venous blood alcohol level at the time of taking the saliva sample with a standard error of ± 0.075 mg/ml.

Friedemann *et al.* (8) reported an average value of 1.10 for 13 saliva alcohol: venous blood alcohol ratios, where the samples were obtained from three subjects 1 to 3 hours following ingestion. The blood alcohol concentrations ranged from 0.052 to 0.126% and the ratios varied from 0.97 to 1.31. In six comparisons on six different individuals charged for driving while under the influence of alcohol, Prag (21) found an average ratio of 1.20, ranging from 1.10 to 1.40. It is not clear whether arterial or venous blood was used. Abels (22), in a study of 50 individuals, found a good correlation between the saliva and venous blood alcohol levels. The experimental evidence indicates that once the period of rapid absorption of alcohol from the stomach into the blood stream and body tissues has passed, the concentration of alcohol in the venous blood may be estimated with reasonable accuracy from the concentration found in saliva.

The analysis of saliva for alcohol offers no special difficulties but infrequently a person may not be able to provide a sample. One volunteer in these tests, after drinking could not produce enough for analysis.

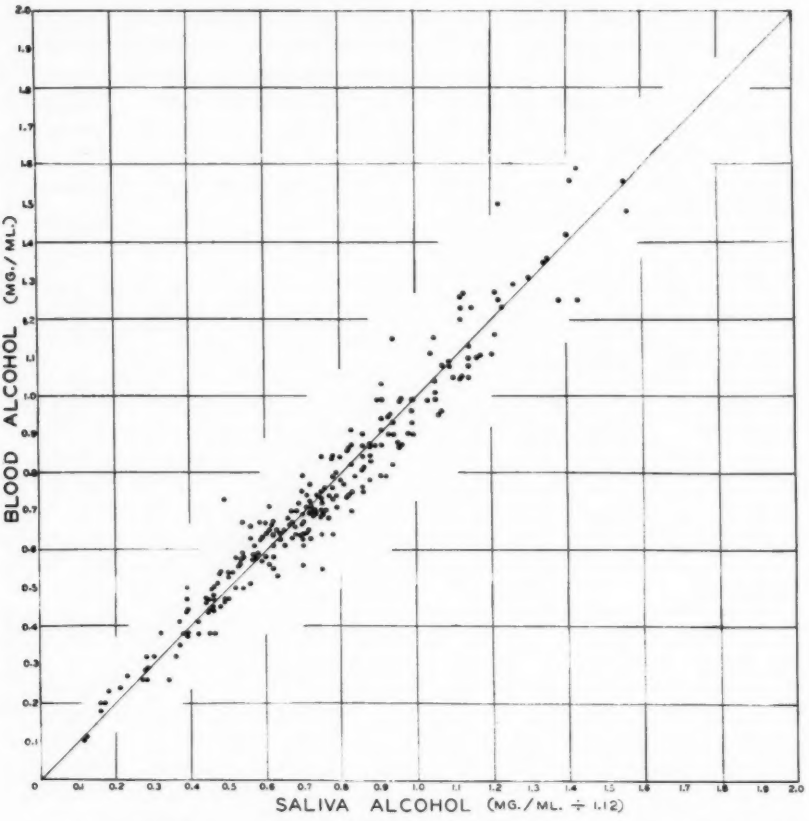

FIG. 2. Relationship between the amounts of alcohol found present in venous blood on direct analysis of blood samples and indirectly by analysis of saliva samples.

Blood and Urine

During preliminary experiments on 16 of the 68 volunteers it was noted that the urine alcohol, divided by an appropriate factor, agreed closely with the venous blood alcohol level at the half-time period of urine accumulation in the bladder. Subsequently, schedules were arranged so that blood samples were taken at a time halfway between the times of voiding. These collections enabled us to compare the alcohol levels in 91 paired samples of urine and venous blood. In 46 of these the concentration of alcohol in the blood, at the half-time period of urine accumulation in the bladder, was estimated by interpolation between the levels found in blood samples taken at known times; in the remaining 45, as indicated above, the times of blood sampling coincided with the half-time period of urine accumulation.

The results are shown in Fig. 3. A high degree of correlation is evident and there is no suggestion that the magnitude of the differences between

the two methods of estimating the venous blood alcohol level is related to the latter. The weighted average ratio of blood alcohol to urine alcohol, obtained by dividing the sum of the blood alcohol concentrations into the sum of the corresponding urine alcohol concentrations, was found equal to 1.24 ± 0.08. There was a slight but non-significant indication that the ratio was higher 45 minutes after drinking than after the lapse of longer periods.

Miles (23) reported a ratio of 1.45 on 80 comparisons of venous blood and urine. In his experiments the subjects were fasting, only small volumes of relatively dilute alcohol solutions were consumed, and the blood alcohol concentrations were mostly less than 0.05%. Carson *et al.* (14), in studies on the possible intoxicating action of 3.2% beer, found the urine alcohol to venous blood alcohol ratio equal to 1.40:1.00. This was based on nearly 150 observations on 36 subjects, the samples being taken 1 hour after a 10-minute drinking period. The urine samples accumulated in the bladder for

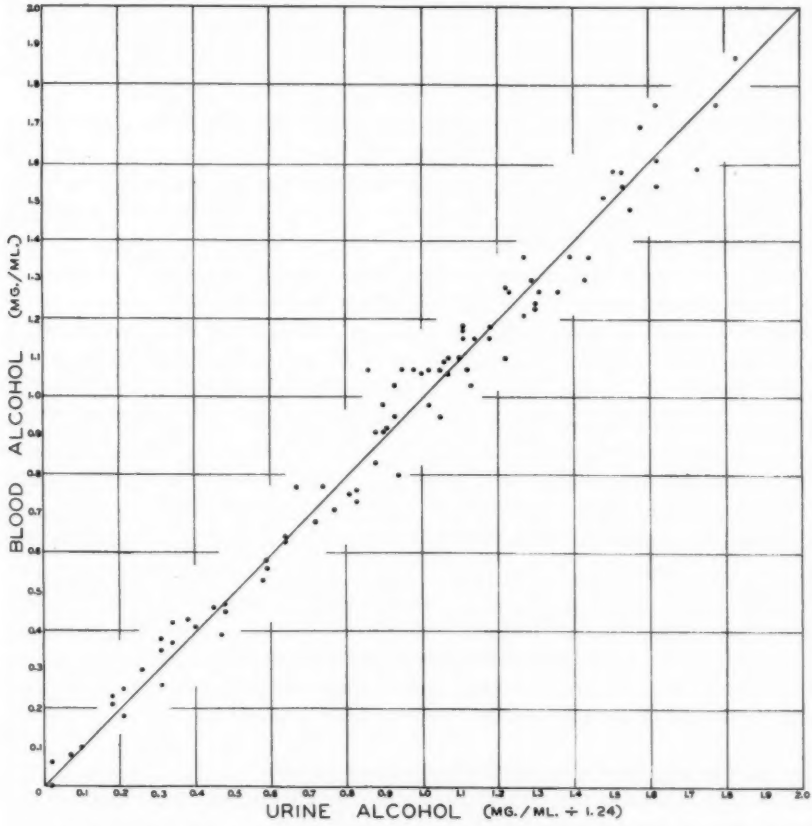

FIG. 3. Relationship between the amounts of alcohol found present in venous blood on direct analysis of blood samples and indirectly by analysis of urine samples.

30 minutes. Southgate and Carter (9) found a ratio of 1.42 based on 42 comparative samples taken from three subjects during the period from 2.5 to 6.5 hours following drinking. Friedemann *et al.* (8) found an average ratio of 1.35 based on 12 comparative urine and venous blood samples, taken 1 to 3 hours following rapid ingestion from three fasting subjects. Several investigators have compared the venous blood and urine alcohol concentrations found in drinking drivers or persons admitted to hospital for acute alcoholism. These are summarized below:

Reference	No. of comparisons	Average UA/BA	Range
Bavis (24)	66	1.17	0.81–1.65
Mozes *et al.* (25)	100	1.16	0.63–1.77
Jetter (26)	372	1.23	1.00–2.30
Ellerbrook *et al.* (27)	76	1.26	0.69–1.71
Prag (21)*	75	1.31	1.00–1.64

*It is not clear whether Prag used venous or capillary blood.

Many of the differences between the results of various workers are due to differences in the experimental conditions. The concentration of alcohol in a sample of urine will be the average for all the urine secreted during the time of accumulation in the bladder. Hence, it may be more or less than the amount in a sample of venous blood obtained at the same time. Haggard *et al.* (10) collected the urine from 11 subjects at ½-hour intervals from 90 minutes to 10 hours after ingestion of 250 cc of undiluted whisky. The ratio of urine alcohol to capillary blood alcohol, averaged for the 198 comparisons, was 1.31; the greatest deviation between the blood alcohol and urine alcohol/1.3 was 0.009%. When the urine was allowed to accumulate in the bladder for longer periods the accuracy of estimating the alcohol concentration in the capillary blood from the amount present in urine decreased. These authors suggest (*a*) that, preferably, only urine accumulated over a 30-minute period in the bladder should be used for the indirect estimation of the concentration of alcohol in (capillary) blood, and (*b*) that urine alcohol/1.3 = blood alcohol. Our results show that, following the period of rapid absorption of alcohol from the stomach and intestines into the blood and tissues, the concentration of alcohol in the venous blood at the half-time period of urine accumulation in the bladder may be estimated with reasonable accuracy from that found in the urine by dividing the latter by 1.24. The period of accumulation in the bladder should be kept within 30 to 60 minutes, if possible.

Rate of Disappearance of Alcohol from Venous Blood

The rate at which the concentration of alcohol in the blood diminishes, designated β, can be determined if samples are taken for analysis after the maximum concentration in the blood has been reached. For example, the

second and third blood samples obtained 88 minutes apart from one subject contained 0.109 and 0.087% alcohol, respectively. Therefore,

$$\beta = \frac{0.109 - 0.087}{88} = 0.00025\% \text{ per minute;}$$

and the rate of decrease per hour,

$$\beta 60 = 0.00025 \times 60 = 0.015\% \text{ (or } 0.15 \text{ mg/ml).}$$

From the data on blood alcohol levels accumulated in these experiments, 110 individual determinations of $\beta 60$ on 53 subjects were made, giving an average value of $0.013\% \pm 0.005$. This compares favorably with the value of $0.015\% \pm 0.0034$ reported by Widmark (28), and Goldberg's figure of $0.014\% \pm 0.0024$ based on experiments with 62 subjects (29). When the $\beta 60$ values were plotted against the venous blood alcohol levels, there was some indication that the rate of disappearance was slightly faster at the higher levels. This is in agreement with Goldberg (29), who reported that an eightfold increase in the quantity of alcohol ingested nearly doubled the rate of disappearance from the blood.

Widmark (28) developed the hypothesis that the ratio of the concentration of alcohol in the *whole body* to the concentration of alcohol in the *circulating blood* is the same as the ratio of water in the entire body to the water content of the blood. This ratio (γ) was found by Widmark to average 0.68 for males. Goldberg (29) reported a value of 0.72 ± 0.08, with a range from 0.59 to 0.97. The weight of ethyl alcohol, expressed in grams, eliminated from the whole body is thus equal to:

$$\gamma \times \beta 60 \text{ (mg/ml), per kg body wt. per hr.}$$

For the average value of $\beta 60$ reported above, the amount eliminated is equal to 88 mg of ethyl alcohol (per kg body wt. per hr) or 0.004 fl. oz of 70-proof distilled spirits (per lb body wt. per hr). While such an estimate is subject to considerable variation, it enables one to determine the approximate amount of alcohol in the body at some time prior to the time of taking a sample of body fluid for analysis. In medicolegal investigations such information may be of some importance.

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ETHYLENE METABOLISM IN TOMATO FRUIT

III. EFFECT OF 2,4-DINITROPHENOL ON RESPIRATION, ETHYLENE EVOLUTION, AND RIPENING[1]

MARY S. SPENCER

Abstract

The role of phosphorylation in fruit ripening was investigated by the introduction of a phosphorylation uncoupling agent, 2,4-dinitrophenol (DNP), into intact fruit. DNP produced a large increase in carbon dioxide evolution by mature green tomatoes, but not by tomatoes in earlier or later stages of maturity. In contrast to its effect on carbon dioxide production, DNP treatment of mature green tomatoes resulted in an immediate depression of ethylene evolution and in failure of the fruit to ripen normally. This suggests a requirement for oxidative phosphorylation for fruit ripening and ethylene production. It was also observed that cell walls of mature green tomatoes treated with the uncoupling agent became wavy and greatly thickened.

Introduction

This paper reports a study of some factors that control respiration in fruit and their effect on ethylene evolution and fruit ripening.

Pearson and Robertson (1) and Millerd, Bonner, and Biale (2) found that the addition of 2,4-dinitrophenol (DNP) to apple and avocado tissue slices or mitochondria resulted in increased respiration of material from preclimacteric but not from climacteric fruit. The latter authors also found that supernatant liquid from centrifugal separation of climacteric avocado mitochondria, when added to mung bean mitochondria in an adenylate-deficient system, produced a maximum oxidative rate and a minimum P/O ratio. This effect was not exerted to any extent by supernatant liquid from preclimacteric fruit. They suggested that mature avocado fruits contained principles which, like DNP, acted to decrease the dependence of respiratory oxidation on coupled phosphorylation. Fruit ripening, then, could be regarded as an uncoupling process, where the supply of adenosine triphosphate would be diminished to low levels, which probably would be inadequate to maintain the metabolic level characteristic of the unripe fruit. However, Marks, Bernlohr, and Varner (3) have recently reported experiments with P^{32} which showed that oxidative phosphorylation reached a maximum in early climacteric fruit and did not disappear until termination of senescence. Other workers (4) found that total high-energy phosphate production paralleled CO_2 production, with a maximum which corresponded to the respiratory peak. These experiments indicate that the climacteric rise in respiration cannot be attributed to the uncoupling of phosphorylation from respiration. Rather, this evidence supports the suggestion of Robertson and Turner (5) that the climacteric rise in fruit respiration might result from the freeing of phosphate acceptors when rapid dephosphorylation occurs to meet demands of protein synthesis in the large cells of full-size fruit.

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Investigators in this field have found that the climacteric rise in respiratory rate was much less with tissue slices (which they used) than with intact fruit. A further difference in behavior was that the slices, especially of preclimacteric fruit, respired more rapidly than an equal amount of tissue in the whole fruit.

To eliminate these difficulties, we introduced DNP into a whole fruit by vacuum infiltration. This made possible measurements of the effect of DNP on ethylene evolution as well as on respiration. (Since the amount of ethylene produced by fruit is very small, no information about the effect of dinitrophenol on the evolution of this gas was obtainable from the use of mitochondria or tissue slices.) Also, in our experiments the effect of DNP on ripening could be observed. If ripening is, in fact, basically an uncoupling process, the DNP should be able to induce chemical changes characteristic of ripening.

Materials and Methods

Tomatoes (*Lycopersicon esculentum*, V 121 variety), which had been shown (6) to exhibit a typical climacteric accompanied by ethylene evolution, were used in these experiments. Selection for maturity was made according to the classification described in the first paper (6) of this series.

The methods for determination of ethylene and carbon dioxide were those reported previously (6). Briefly, a stream of purified air was passed at constant rate through a flowmeter into a respiratory chamber, which contained the fruit, and then out to a tower of barium hydroxide solution for carbon dioxide absorption or into mercuric perchlorate solution for ethylene absorption. Carbon dioxide was determined by Biale's and Shepherd's (7) modification of Truog's (8) method. Ethylene was determined manometrically by the procedure of Young, Pratt, and Biale (9). At least two runs were made for each variation in experimental conditions and typical data are presented.

To introduce solutions into the fruit, a vacuum infiltration technique similar to that of McCready and Hassid (10) for leaves was used. Tomatoes are particularly suitable for the application of this technique because their skins are practically impermeable to gases, and any exchange of gas (or liquid) is made almost entirely through the stem scar (11, 12).

The fruit was placed in a vacuum desiccator and either inverted in a dish of the solution to be infiltrated, or placed upright with a measured amount of the solution resting on the stem scar. One outlet from the desiccator was an air bleed and the other was connected to a water aspirator. Over a period of 10 to 20 minutes a vacuum of about 9 inches of mercury was drawn. This vacuum was maintained for 5 minutes, and gas bubbles could be observed escaping from the stem scars. When the pressure was returned slowly to atmospheric, the solution was drawn into the tomatoes. Figure 1 shows the penetration of a glucose-C^{14} solution introduced by this method. Visual observations showed that dye and DNP solutions entered to a similar extent

PLATE I

FIG. 1. Radioautograph of tomato infiltrated with a glucose-C^{14} solution.

to the glucose solution. Flooding of the vascular system was avoided when the amount of solution infiltrated was limited to $\frac{1}{4}$ ml per 100 g of tomato. Microscopic examination and respiration measurements revealed no damage to cells from infiltration.

Results and Discussion

Water Infiltration

To determine whether the mere process of vacuum infiltration affected normal ripening and respiratory behavior, tomatoes were infiltrated with water. The results are recorded in Fig. 2. No deviation from the normal (6) gas production and ripening behavior was observed: respiration and ethylene evolution were low when the fruit was mature green. With the initiation of ripening, respiration rose from the preclimacteric minimum to the climacteric maximum, and ethylene evolution also increased rapidly. Subsequent to the climacteric peak, both the respiratory rate and ethylene production dropped steadily as the fruit passed into senescence. No disturbance of the normal behavior occurred when tomatoes were infiltrated either in the early stages of the climacteric or immediately after the climacteric peak had been passed. The texture and color changes of normal ripening took place, with no difference in the areas that had been reached by the infiltrated water as compared with those that had not.

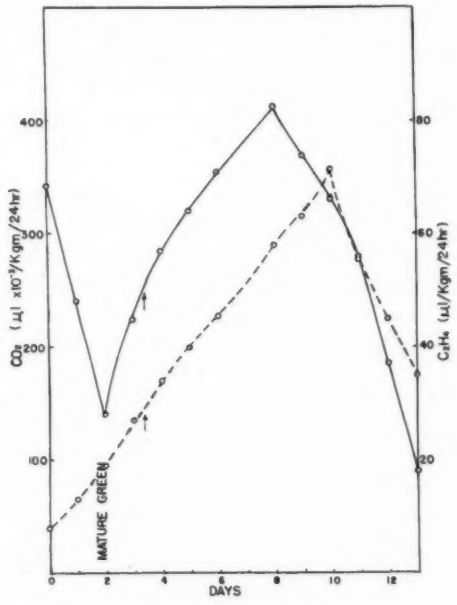

FIG. 2. Gas evolution of tomatoes infiltrated with water. Arrows indicate time of infiltration. (Solid line, CO_2; broken line, C_2H_4).

Effect of DNP on Respiration

Two or more runs were made for each variation in experimental conditions, and the effects of DNP were reproducible for each variation.

Trial runs showed that the respiration of DNP-infiltrated, mature green tomatoes increased with increasing concentrations of solution up to 0.5 g DNP/l., but gradually decreased to normal at higher concentrations. Robertson, Wilkins, and Weeks (13) observed a similar effect with carrot disks. The stimulatory effect was evident even in the first carbon dioxide collection after infiltration, and lasted longer with the higher concentrations of DNP.

When young tomatoes were infiltrated with DNP (0.3 g/l. or 0.4 g/l.) carbon dioxide immediately rose to its normal (6) preclimacteric peaks. Ethylene evolution continued at the low levels usual for this stage of maturity. DNP apparently accelerated the increase in carbon dioxide production, but did not affect the maximum levels of gas production.

DNP treatment of mature green fruit resulted in a rapid increase in carbon dioxide production (Fig. 3). Carbon dioxide reached a peak within 2 days after infiltration and before the tomatoes showed the color and texture changes that usually accompany the peak in respiration. (Untreated tomatoes of this variety normally achieve a respiratory peak and become partially colored ("medium turning") 6 days after reaching the mature green stage.) In

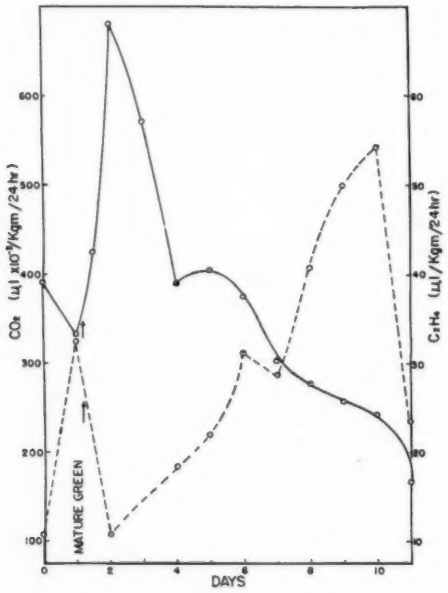

FIG. 3. Gas evolution of tomatoes infiltrated with DNP (0.45 g/l.). Arrows indicate time of infiltration. (Solid line, CO_2; broken line, C_2H_4.)

contrast to carbon dioxide, ethylene evolution decreased immediately after infiltration with DNP, then gradually rose to a peak at the advanced turning stage where the ethylene maximum occurs for untreated fruit.

The initial effect of DNP on mature green tomatoes was to depress ethylene evolution and to increase carbon dioxide evolution. Since DNP uncouples phosphorylation from oxidation, this suggests that the production of ethylene is an energy-requiring process. Also, the mechanism of formation of this gas appears to be distinct from that of carbon dioxide.

Present methods of ethylene analysis are not sensitive enough to allow determination of ethylene evolved from mitochondria or tissue slices. Whole fruit must be used, and gas evolution from untreated portions of the fruit continues as a background for the respiration of the treated portions. This may account for the increase in ethylene evolution that took place several days after infiltration, and possibly for the small rise in carbon dioxide production on the 5th day.

With climacteric and postclimacteric tomatoes, concentrations of DNP of 0.3 to 0.5 g /l. had no effect on the evolution of either carbon dioxide or ethylene, other than a slight increase in carbon dioxide evolution immediately after infiltration.

Thus, treatment with DNP resulted in markedly increased carbon dioxide evolution only when tomatoes were in the mature green stage. (This work with whole fruit corroborates the results obtained by Pearson and Robertson (1) with apple disks, and by Millerd and co-workers (2) with avocado tissues and mitochondria.) Ethylene production did not increase along with carbon dioxide evolution when mature green tomatoes were infiltrated with DNP. Rather, a depression in ethylene production occurred immediately after infiltration.

Effect of DNP on Ripening

These experiments with whole tomatoes permitted observation of the effects of DNP on fruit ripening. When young or mature green tomatoes were treated with DNP, they failed to ripen normally. Where the uncoupling agent had penetrated the fruit, it rapidly became soft in texture and chlorophyll destruction occurred. However, the resulting texture was spongy, rather than that typical of a ripe tomato, and visual observation indicated that lycopene and other carotenoids did not develop. (Marks and co-workers (3) have also reported that fruit fails to ripen normally when treated with DNP.) This behavior is consistent with the concept of ripening as an energy-requiring process. Also, there is the possibility of a relationship between the depression in ethylene production and the inability to ripen. Parts of the fruit which had not been reached by DNP, and which presumably accounted for the later rise in ethylene evolution, ripened normally.

Microscopic examination showed that the nuclei of all DNP-treated cells were intact and well shaped, and that the cytoplasms were capable of plasmolysis. However, interesting changes had occurred in the cell walls of these

cells. As shown in Fig. 4, shortly after DNP treatment the cell walls began to thicken and assume a wavy appearance. Tomatoes examined 1 day after DNP infiltration possessed distinctly wavy and greatly thickened walls. Cells of ripe tomatoes infiltered with DNP and of green or ripe tomatoes infiltered with water were normal in appearance. Little work has been done on the changes in fruit cell walls during ripening (14), but the fact that an uncoupling agent produced such marked alterations in the cell walls of unripe fruit is an interesting phenomenon.

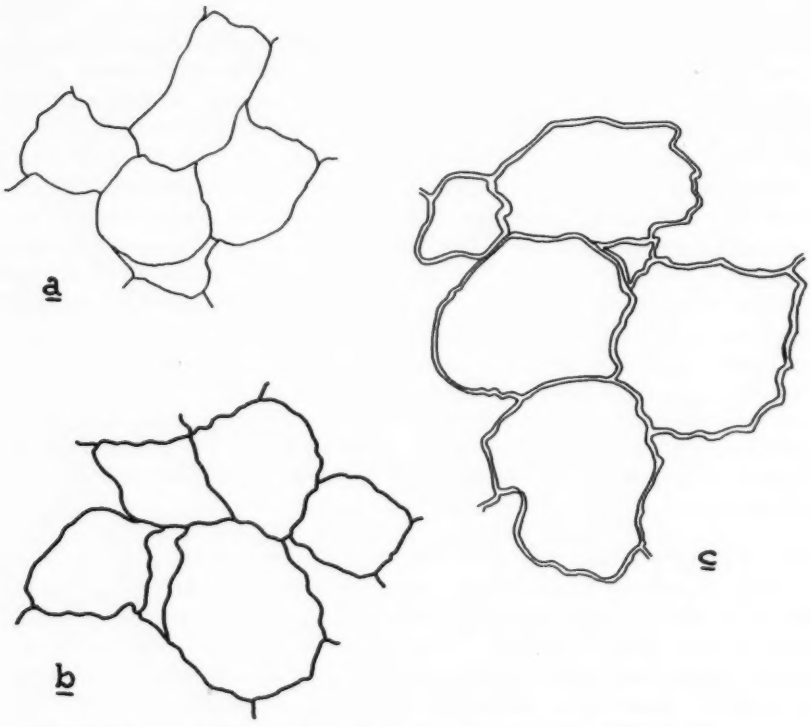

FIG. 4. Camera lucida drawings of the microscopic appearance of cells of mature green tomatoes: (*a*) untreated cells, (*b*) cells 2½ hours after infiltration with DNP (0.45 g/l.), (*c*) cells 24 hours after infiltration with DNP (0.45 g/l.). (Magnification: ×46.)

Acknowledgments

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REACTION OF THE THYMUS TO TRAUMA IN DRUM-RESISTANT RATS[1]

Z. HRUZA AND F. CHYTIL

Abstract

The reaction of the adrenals and thymus to trauma in a Noble–Collip drum was studied in control rats and in rats that had developed trauma-resistance by increasing traumatization in this drum. Although the reaction of the adrenals is the same in both groups as determined by the ascorbic acid and cholesterol content, the thymus decreases in weight only in control animals exposed to trauma for the first time. Thymus regresses in trauma-resistant rats only after a mortal dose of trauma. Trauma-resistant rats have heavier adrenal glands than control animals. This difference is more expressed in males than in females. Catabolic doses of cortisone cause a lesser loss in thymus weight in resistant animals than in control rats. After sterile abscess, the loss of thymus weight was only slightly smaller in trauma-resistant rats.

Introduction

It is possible to achieve considerable resistance to trauma in a Noble–Collip drum in rats and guinea pigs by repeated increasing exposure of animals to this kind of trauma (14). The metabolic response to trauma in trauma-resistant animals has been studied by a number of authors (12, 11, 9, 1, 2, 3). It was shown by Noble and Collip (16) that even adrenalectomized rats can be adapted to trauma in a Noble–Collip drum, although to a lesser extent. Consequently the adrenal glands are not completely indispensable for the development of trauma-resistance. The role of the adrenals after resistance has already developed has not been studied. In the present paper the reaction of the adrenal glands and thymus was studied in trauma in the Noble–Collip drum in control and trauma-resistant animals.

Experimental

Adult rats (Wistar strain) were fed on a standard laboratory diet containing 52.8 Cal% carbohydrates, 25.5 Cal% proteins, and 21.7 Cal% fats. The detailed composition is given in a previous paper (4). Some of the animals were made trauma-resistant by using increasing doses of mechanical energy, as described by Noble (14) and applied in a previous paper (1). A Noble–Collip drum with two prisms rotating 45 times/minute was constructed according to Noble and Collip (15). Trauma was applied in doses according to the weight of the animal and expressed in traumatizing mechanical energy acting on 1 g of the animal (5). Rats were adapted to trauma during 3 weeks. Trauma-resistant rats survived a dose considerably larger than the lethal dose for control animals (30 minutes in the drum, which corresponds to a traumatizing energy of about 1440 kg-m*/g body weight). In view of the fact that the weights of individual animals in the experimental groups were

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*kg-m = kilogram-meter of energy.

approximately the same, the values of traumatizing mechanical energy are proportional to the time spent by the animal in the drum, as expressed in minutes. Sterile abscess was made by subcutaneous injection of 0.2 ml turpentine oil in the left back paw.

The weight of the thymus was determined after the gland was fixed in 10% formol. Total nitrogen was determined by micro-Kjeldahl according to Ma and Zuazaga (8). Adrenal glands were weighed immediately after they had been removed from the body. Ascorbic acid in the glands was determined according to Kingsley and Schaffert (6), cholesterol according to Koch and Hanke (7). Results were evaluated statistically using the *t* test.

Results

The Effect of Trauma on Thymus Weight and the Ascorbic Acid and Cholesterol Content in the Adrenal Glands

A group of control and trauma-resistant rats were subjected to the effects of mechanical energy 460 kg-m/1 g wt. (about 10 minutes in the drum). Experimental animals were usually killed in groups of six after 0, ½, 1, 2, 3½, 6, 24, and 48 hours. The weight of the thymus and adrenals and the ascorbic acid and cholesterol content in the latter were determined. Results are given in Fig. 1. It is evident from the figure that thymus weight decreases significantly only in the control group after trauma but not in the trauma-resistant one. The concentration of ascorbic acid in the adrenals, on the other hand, decreases and then increases at about the same speed in both groups.

There is the same fall of adrenal cholesterol in both groups. The amount of ascorbic acid released from the adrenals during the first 2 hours after trauma is higher in trauma-resistant rats.

These experiments were repeated in several series of control and trauma-resistant rats with the same results. Determination of the total thymus nitrogen also confirmed that in trauma-resistant rats the thymus does not atrophy after trauma (Table I).

As the trauma applied to the trauma-resistant rats might have been too small, doses of trauma three times that used in the previous experiment were applied to trauma-resistant rats, i.e. 1440 kg-m/g (about 30-minute rotation). In adapted animals this dose does not cause death while in control animals it is well above the lethal dose. Not even this trauma causes decrease in thymus weight after 24 or 48 hours as is shown in Table I and Fig. 1.

When the dose of trauma in trauma-resistant rats was increased to LD 18 and LD 62 (i.e. 45 and 60 minutes) there was a regression in thymus weight (Fig. 2, Table I).

Adrenal Weight in Control Animals and in Trauma-Resistant Rats before and after Trauma

Adrenal weights were considerably higher in trauma-resistant rats than in control animals. This is especially the case in males (19.5 ± 0.7 and 14.2 ± 0.4

mg/100 g, $P<0.001$), less so in females (33.4 ± 1.5 and 29.2 ± 1.2 mg/100 g, $P<0.05$). The resting values of ascorbic acid and cholesterol concentrations in the adrenals were the same in both groups and did not differ statistically.

The Sensitivity of the Thymus of Trauma-Resistant Rats to Cortisone and Turpentine Abscess

The above results show that the thymus does not regress after trauma that is not mortal for the trauma-resistant rats. It is not clear if the target organ is more resistant to corticoids or if the trauma-resistant rats do not release the same amount of corticoids as do the controls. For these reasons cortisone acetate (cortisone acetate Continental Pharma, S.A. Belgo-Canadienne) was administered via the intraperitoneal route in two doses, one 6 hours after the other, a total of 8 mg/100 g body weight. The weight

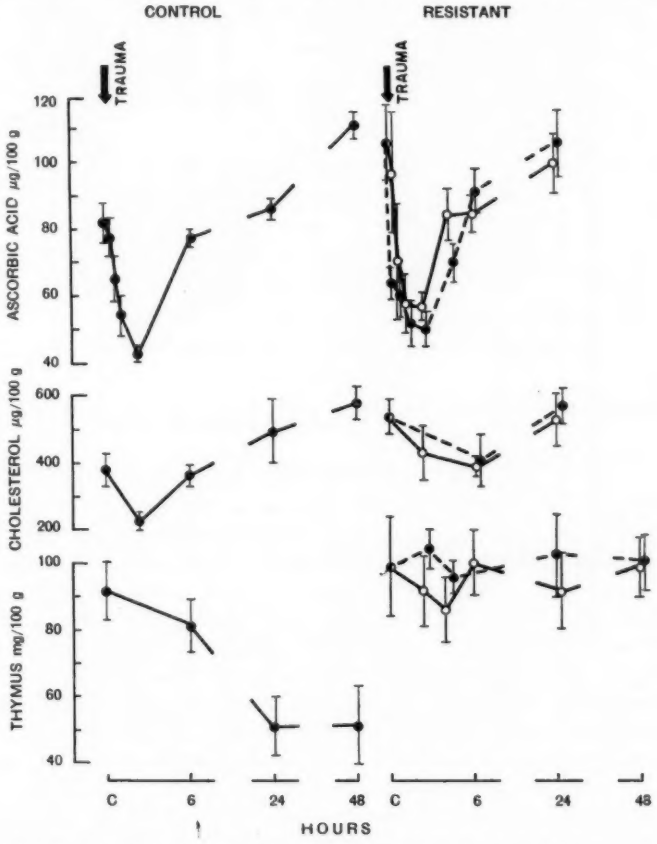

FIG. 1. Ascorbic acid and cholesterol in the adrenals in control (●———● 10 min trauma) and resistant (10 min ○———○ and 30 min ●– – –●) rats and thymus weight after trauma (averages and S.E.).

TABLE I

Changes in weight of the thymus after trauma in the Noble–Collip drum

	Dose of trauma	Hours after trauma	Body weight	Sex	No. of rats	Thymus, mg/100 g body weight				Total N in thymus, mg/100 g body wt.			
						Mean	± S.E.	Fall in %	Significance	Mean	± S.E.	Fall in %	Significance
Control rats	0		294		6	91.4	±7.9						
	10 min (464 kg-m/g)	24	307	♂	6	51	±8.7	42.2	$P<0.01$				
	10 min (464 kg-m/g)	48	312		5	51.6	±12.1	43.6	$P<0.02$				
	0		162		7	250.4	±10.9						
	8 min (464 kg-m/g)	24	161	♀	8	209	±10.5	16.5	$P<0.02$				
	0		227		14	139	±9.5			3.06	±0.23		
	5 min (232 kg-m/g)	48	221	♂	10	137.7	±9.5	1.7	Non-sig.	2.81	±0.20	8.1	Non-sig.
	10 min (464 kg-m/g)	24	229		8	111.4	±6.3	19.8	$P<0.05$	2.40	±0.18	21.6	$P<0.05$
	10 min (464 kg-m/g)	48	219		10	111.3	±7.9	20	$P<0.05$	2.41	±0.17	21.2	$P<0.05$
Trauma-resistant rats	0		272		8	106.2	±8.9						
	10 min (464 kg-m/g)	24	274	♂	7	100	±9.1	5.6	Non-sig.				
	0		283		6	98.8	±15.5						
	10 min (464 kg-m/g)	24	283	♂	6	91.5	±11.3	7.3	Non-sig.				
	0		212		6	118.6	±10.1			2.41	±0.25		
	30 min (1392 kg-m/g)	24	235	♂	7	105.1	±8.1	11.2	Non-sig.	2.42	±0.25	0	Non-sig.
	0		213		9	113.4	±9.4			2.26	±0.22		
	30 min (1392 kg-m/g)	24	213	♂	8	117.8	±13	0	Non-sig.	2.54	±0.25	0	Non-sig.
	30 min (1392 kg-m/g)	48	223		8	113.7	±8.6	0	Non-sig.	2.15	±0.28	4.7	Non-sig.
	0		227		15	128.6	±7.8			2.84	±0.18		
	30 min (1392 kg-m/g)	48	208		8	115.8	±9.4	11.8	Non-sig.	2.43	±0.27	14.3	Non-sig.
	45 min (2028 kg-m/g)	48	200	♂	9	84.8	±9.5	34	$P<0.01$	1.59	±0.23	44.0	$P<0.001$
	60 min (2784 kg-m/g)	48	213		6	87.1	±13.6	32.1	$P<0.02$	1.89	±0.29	33.5	$P<0.01$

of the thymus was determined together with the nitrogen content 48 hours later. The same indicators were taken in control and trauma-resistant groups not receiving cortisone. Results are given in Table II. It can be seen from the table that cortisone causes a decrease in thymus weight and in total nitrogen in both control and trauma-resistant animals. The decrease in thymus weight, however, is larger in the control group (the decrease in thymus weight expressed in per cent of the original weight is significantly greater in the control than in the trauma-resistant group).

Finally we estimated the sensitivity of the thymus to other kinds of stress, i.e. sterile abscess. As shown in Table II thymus regresses in both groups and a slightly greater regression (not significant difference) was found in the control group.

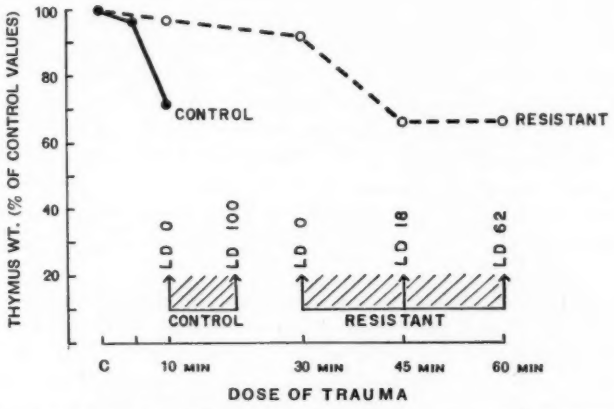

FIG. 2. Loss of weight of the thymus in control (●———●) and trauma-resistant rats (○- - -○) at different doses of trauma.

Discussion

The weight of the thymus and its nitrogen content do not decrease in trauma-resistant rats after trauma in the Noble–Collip drum except after mortal doses. In control animals, after the same dose of trauma there is atrophy of the thymus. It is not clear why this is so.

In wild rats a fall in ascorbic acid content in the adrenals was not observed after acute cold or auditory stress (21). Vogt (19) showed that an emotional stimulus, if repeated after rats were trained for 6 consecutive days, entirely lost its effect on adrenal ascorbic depletion. Nicholls and Rossiter (13) recently claimed that, in rats adapted to cold, the acute pituitary–adrenal response to further stress is decreased. In our results in trauma-resistant rats it was found that, on the contrary, trauma-resistant rats release more total ascorbic acid from the adrenals after trauma than control animals. This is probably the maximal amount of ascorbic acid which can be released because after 200% greater trauma in resistant rats, the same fall in ascorbic acid content was observed. The differences between the present and the

TABLE II

Loss of weight of the thymus after cortisone treatment or sterile abscess in male rats

Group	Treatment	Hours after treatment	Body wt.	No. of rats	Thymus, mg/100 g body weight				Total N in thymus, mg/100 g body weight			
					Mean	± S.E.	Fall in %	Significance	Mean	± S.E.	Fall in %	Significance
Control	0		230	6	139.7	±14.1			2.90	±0.36		
	8 mg cortisone/100 g	48	224	6	77.4	±5.2	44.6	$P<0.01$	1.56	±0.16	46.2	$P<0.02$
	0		200	8	165.6	±18.0			3.95	±0.44		
	8 mg cortisone/100 g	48	188	8	94.8	±9.9	42.8	$P<0.01$	2.11	±0.26	46.5	$P<0.01$
Resistant	0		213	9	113.4	±9.4			2.26	±0.22		
	8 mg cortisone/100 g	48	213	9	89.3	±9.4	21.2	Non-sig.	1.72	±0.13	23.9	$P<0.05$
	0		261	15	93.3	±6.2			2.22	±0.22		
	8 mg cortisone/100 g	48	250	15	62.7	±4.4	32.8	$P<0.001$	1.36	±0.09	38.7	$P<0.01$
Control	Calc. in % of control values (expt.	48		14	56	±3.6%			53.6	±4.3%		$P<0.05$
Resistant	connected)	48		24	71.7	±4.3%		$P\doteq0.01$	67.2	±3.9%		
Control	0		227	14	139.0	±9.5			3.06	±0.23		
	Turpentine abscess	72	223	8	67.4	±8.1	51.5	$P<0.001$	1.37	±0.16	55.2	$P<0.001$
Resistant	0		227	15	128.6	±7.8			2.84	±0.18		
	Turpentine abscess	72	201	9	84.5	±7.3	34.2	$P<0.01$	1.62	±0.22	42.8	$P<0.001$
Control	Calc. in % of control values	72		8	48.5	±5.8			44.8	±5.2%		
Resistant		72		9	65.8	±5.6		$P\doteq0.05$	57.2	±7.7%		Non-sig.

above-mentioned experiments can be explained by the much greater stress used here. According to earlier data (17) there is a correlation in the corticoid and ascorbic acid release from the adrenals. Recently Slusher and Roberts (18) found that the ascorbic acid is released quantitatively from the adrenals before the depletion of adrenal corticoids. For this reason it is difficult to relate the amount of ascorbic acid released from the adrenals directly to the amount of corticoids. One can only say that the adrenals in both our groups are activated.

The fact that the thymus does not atrophy in trauma-resistant rats indicates that the target organ—thymus—shows a decreased sensitivity to corticoids or to other factors, acting during trauma, that might have an effect on the thymus. McKenna and Zweifach (10) found that the reticulo-endothelial system of trauma-resistant rats reacts less to catabolic doses of cortisone. Thus the decreased sensitivity of the thymus to corticoids as shown in this paper and the same phenomenon in the reticulo-endothelial system might be due to a similar mechanism. The results with sterile abscess in the rat show that there is little difference in the thymus response between control and drum-resistant rats. The resistance of the thymus is probably related to drum trauma and not to other kinds of stresses. At least two mechanisms concerned with the resistance of thymus may participate, the smaller sensitivity of the target organ to corticoids or to other stimuli acting after the trauma.

A smaller adrenal hypertrophy in trauma-resistant females than in trauma-resistant males was found. These results are in agreement with those of Woods (20), who in wild rats had observed a greater adrenal hypertrophy than in domesticated rats after long-term exposure to cold. In both experiments there was a higher resting adrenal weight in the group which had a smaller reaction after stress. Thus the smaller hypertrophy could be explained by heavier adrenals in females.

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STUDIES ON THE PRESERVATION OF BLOOD

VII. THE INFLUENCE OF INOSINE ON THE METABOLIC BEHAVIOR OF THE ERYTHROCYTE DURING THE PRESERVATION OF BLOOD IN THE COLD[1]

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With the technical assistance of SHEILA GOSSELIN

Abstract

Inosine, like other purine nucleosides when added to preserved blood specimens, induces a resynthesis of organic phosphate esters in the erythrocytes. Apart from the difference in rates of reaction, the metabolic reconstitution of the cells is the same at 37° and 4° C. The reconstitution of the esters occurs rapidly even at 4° C, and the higher the concentration of inosine added, the more prolonged is the maintenance of the esters. When inosine was added repeatedly to blood samples during storage, a phase of synthesis was induced with each addition of nucleoside. The capacity of the erythrocytes to resynthesize 2,3-diphosphoglyceric acid (DPG) remained normal regardless of the storage age of the sample but the capacity to replenish ATP decreased with the duration of storage. Addition of inosine at the end of the third week of cold storage can effect a high degree of restoration of metabolic viability in the cells at 4° C within 24 hours.

The preceding two communications (1, 2) of this series dealt with the influence of adenosine and inosine on the metabolism of the erythrocytes during preservation of blood in the cold. The present paper concerns the influence of the concentration of inosine on the metabolic activity of the preserved cells. The aims of the study were: (*a*) to determine the concentrations of inosine required for the preservation of the red cells for various periods of time at 4° C; (*b*) to ascertain the most favorable time to add the inosine to the blood specimen during the storage period.

Experimental

Reagents

The biochemical reagents were obtained from Nutritional Biochemicals Corporation.

Methods

The procedure for the collection and preservation of blood, and the details of the analytical methods, were as described in paper No. VI of this series (2).

Procedures and Results

1. Effect of Various Concentrations of Inosine on the Metabolic Activity of Erythrocytes at 37° C

Specimens of human blood were incubated at 37° C to estimate the rate at which added inosine is utilized by the erythrocytes at this temperature.

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Contribution from the Department of Biochemistry, McGill University, Montreal, Quebec. This study was supported by the Defence Research Board of Canada, grant No. 9350-01, Project D 50-93050-01.

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Four lots of saline-washed erythrocytes were resuspended in a medium containing isotonic saline, glucose (in final concentration of 600 mg%), penicillin (1,000,000 units), and inosine in a final concentration of 100, 200, 400, and 700 mg%, respectively. The specimens were incubated for 53 hours and, at various intervals, samples were removed aseptically for the estimation of glucose, ribose, lactate, and inorganic phosphate. In Fig. 1 are represented the quantity of ribose utilized, lactate produced, and inorganic phosphate liberated during the period of incubation.

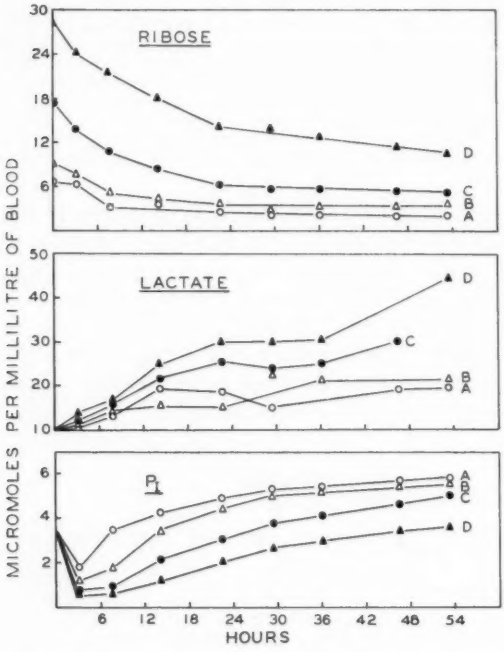

FIG. 1. Utilization of ribose and production of lactate and inorganic phosphate by human erythrocytes at 37° C.
A. Erythrocytes in medium (see page 69) containing 100 mg% of inosine.
B. Erythrocytes with 200 mg% of inosine.
C. Erythrocytes with 400 mg% of inosine.
D. Erythrocytes with 700 mg% of inosine.

It is evident that esterification of inorganic phosphate occurred from the beginning of incubation with all four concentrations of inosine. The synthetic activity, however, predominated for only a few hours and then gave way to hydrolysis. The onset of hydrolysis occurred earliest and at the most rapid rate in the specimen with the lowest concentration of inosine and was slowest in the sample with the highest concentration.

2. Effect of Various Concentrations of Inosine on the Metabolic Activity of Preserved Erythrocytes in ACD Medium at 4° C

A quantity of blood from one donor was collected in the acidified citrate–dextrose (ACD)[4] medium and divided into four lots, A, B, C, and D. Sterile inosine was added to each specimen to give a concentration of 100, 250, 500, and 800 mg%, respectively. The specimens were then stored at 4° C for a period of 38 days. At intervals, a sample was removed aseptically from each lot for estimation of glucose, ribose, and inorganic phosphate. The amount of glucose and ribose utilized during the storage period is indicated in Fig. 2, and the concomitant behavior of the phosphate fractions, in Fig. 3.

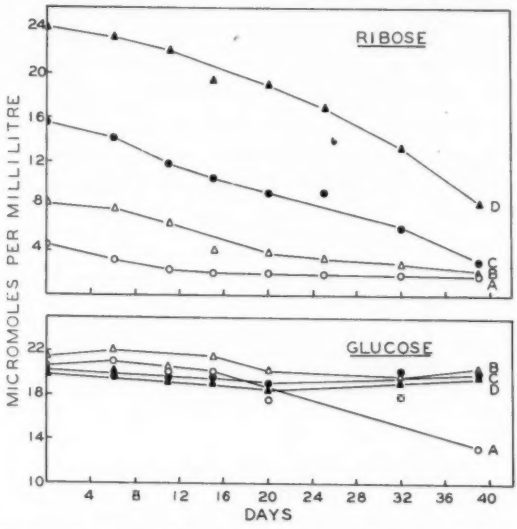

FIG. 2. Utilization of ribose and glucose by human erythrocytes during storage in ACD medium at 4° C.
A. Specimen with inosine, 100 mg%.
B. Specimen with inosine, 250 mg%.
C. Specimen with inosine, 500 mg%.
D. Specimen with inosine, 800 mg%.

The rate of utilization of ribose was more rapid with higher concentration of the inosine. The unused residue of about 2 micromoles per ml in samples A and B appears to represent pentose which is inaccessible for metabolism.

The inhibition of glucose utilization by inosine, as noted in a previous study (2), was again observed. Thus in the specimen with the lowest concentration of inosine the utilization of glucose remained negligible until the

[4]The following abbreviations are used throughout this paper: CD, isotonic citrate–dextrose medium (1.80 g sodium citrate and 1.47 g dextrose per 100 ml of solution, pH 7.4); ACD, acidified citrate–dextrose medium (0.44 g citric acid, 1.32 g sodium citrate, and 1.47 g dextrose per 100 ml, pH 5.5; when mixed with blood, gives pH about 7); P_I, inorganic phosphate; P_L, labile phosphate, mainly ATP, adenosine triphosphate; P_S, stable organic phosphate, mainly DPG, 2,3-diphosphoglycerate; P_H, 'hydrolyzable' phosphate, mainly hexose-6-phosphate.

ribose approached depletion about the 15th day. In the other specimens, in which a fair supply of pentose still remained, there was virtually no utilization of glucose by the cells throughout the 38-day period.

The influence of various concentrations of inosine in maintaining the organic phosphate esters in the erythrocytes during storage is indicated in Fig. 3. Comparison of Figs. 2 and 3 shows that when the concentration of inosine (ribose) reached a low level the organic phosphate esters underwent hydrolysis, as reflected by the increase in the concentration of inorganic phosphate (P_I). It is noteworthy that, in the specimen with the lowest concentration of inosine (100 mg%), the breakdown of organic phosphate began about the 6th day of storage; in the specimen with 250 mg%, about the 20th day, and with 500 mg%, it was delayed until the 32nd day. In the specimen with 800 mg% of inosine the level of the organic phosphates remained unchanged throughout the entire period. The change in the concentration of P_I reflects chiefly the change in the concentration of DPG, which is the organic phosphate present in largest concentration in the erythrocyte. It is significant that, in the specimens with the 500 and 800 mg% of inosine, the ATP was maintained at the normal concentration.

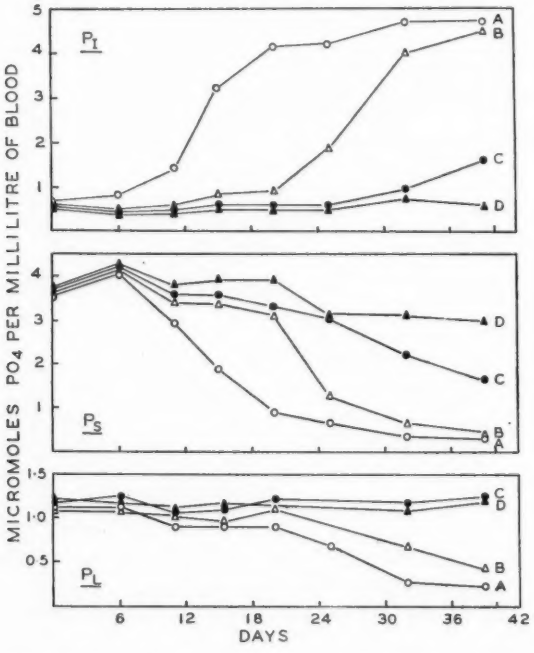

FIG. 3. Behavior of phosphate fractions in blood preserved in ACD medium at 4° C. A, B, C, and D as under Fig. 2.

3. Effect of Inosine when Added Repeatedly to Preserved Blood during the Storage Period

It was of interest to study the influence of inosine on the regeneration of the organic phosphate esters when added to the blood specimen at various times during the storage period. Four specimens of blood from one donor were collected in ACD medium and stored at 4° C for 112 days. To one specimen A, sterile inosine was added immediately to give a concentration of 800 mg%. At 3-week intervals thereafter (indicated by times *b*, *c*, and *d*, in Figs. 4 and 5) the same quantity of inosine was added successively to the remaining preserved specimens B, C, and D, respectively. Periodically, a sample was removed from each of the specimens for estimation of glucose, ribose, and the three phosphate fractions. The curves in Fig. 4 indicate the comparative rates of utilization of glucose and ribose by the cells in the various specimens.

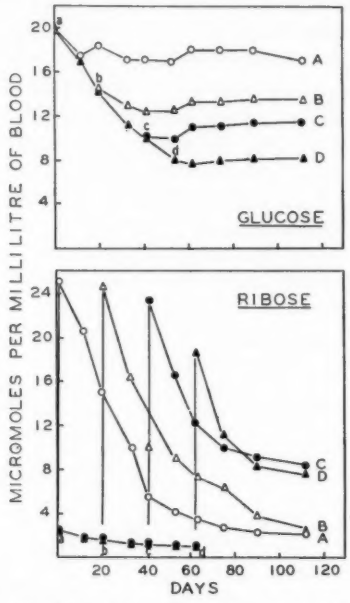

FIG. 4. Comparative utilization of glucose and ribose by addition of inosine to human erythrocytes preserved in ACD medium at 4° C.
A. Inosine added at beginning of storage.
B. Inosine added on 21st (*b*) day.
C. Inosine added on 42nd (*c*) day.
D. Inosine added on 63rd (*d*) day.

The utilization of glucose continued until the time when the nucleoside was added. It is noteworthy that a limited utilization of glucose occurred during the first week in the 'control' specimen to which inosine had been added at the beginning of storage. However, with the production and accumulation of lactic acid, and the consequent increase in the hydrogen-ion concentration, the glucose utilization was brought to a standstill by the end of the second week.

It is significant that the rate of utilization of ribose was about the same in all instances irrespective of the storage age of the specimen when the inosine was added.

The influence of the added inosine on the concentration of the various phosphate fractions of the erythrocytes is indicated in Fig. 5.

In the case of the 'control' specimen A, to which inosine had been added at the start, the concentration of inorganic phosphate remained comparatively low until about the 40th day. From then on, the concentration of P_I increased rapidly with the progressive hydrolysis of the organic phosphate esters. In the other specimens the breakdown of ester phosphate proceeded from the beginning of storage, and the concentration of P_I in these specimens usually reached the maximum about the 20th day. Upon the addition of inosine at the times indicated in Fig. 5 by *b*, *c*, and *d*, respectively, resynthesis of organic phosphate began, and the concentration of P_I, in each case, rapidly declined to the original level. However, the period of reconstitution was relatively short-lived because of the rapid depletion of the supply of nucleoside, and was followed by a progressive breakdown of the esters. In this experiment, as in the preceding one (Fig. 3), the inverse relationship between the change in the concentration of DPG and that of P_I is clearly evident. During the period of regeneration of the esters in the presence of inosine, the concentration of DPG in some instances rose above the original level. The ATP, regenerated in preserved blood specimens in the presence of inosine, never

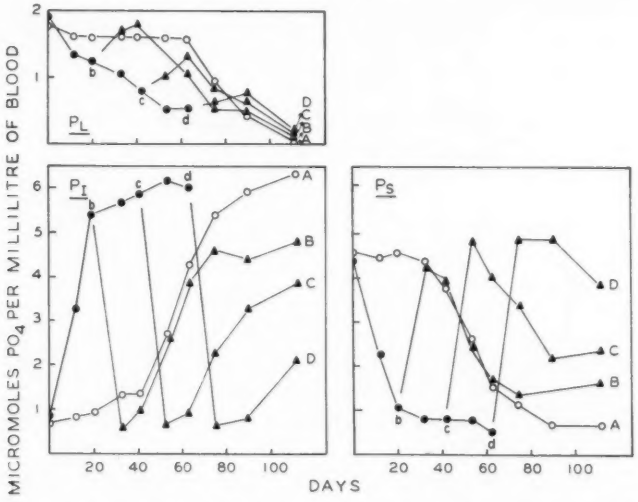

FIG. 5. Resynthesis of organic phosphate esters induced by repeated addition of inosine.
A. Inosine (800 mg%) added at the beginning of storage.
B. Inosine added on the 19th day.
C. Inosine added on the 41st day.
D. Inosine added on the 63rd day.

rises to higher than the original normal level. The maximum degree of regeneration occurs in specimens preserved under favorable conditions (4° C) for not longer than three weeks. Restoration of metabolism can be effected also in older specimens, but the capacity of the cells to regenerate ATP diminishes with the duration of storage. Thus in our studies the magnitude of the regeneration of ATP was relatively slight in the specimen D to which the nucleoside was added at the end of the ninth week. ATP was maintained at the highest level in the 'control' specimen to which inosine was added at the beginning of the storage period. The addition of the nucleoside at the start, therefore, tends to retard the changes that occur during storage under ordinary conditions. It is of interest that inosine can induce the resynthesis of DPG to the normal concentration in cells that have been stored in the cold for 12 weeks or longer (see Fig. 5, curve D).

4. Influence of Repeated Addition of Inosine to Preserved Blood Samples

Blood from one donor was collected into ACD medium and divided into three portions, A, B, and C. To portion A, inosine was added immediately to give a concentration of 400 mg%. The three lots were then stored at 4° C. At the end of the third week, inosine (400 mg%) was added to lots B and C (times indicated by *b* and *c*, in Figs. 6 and 7). To lot C, further additions of inosine (400 mg%) were made at intervals of 3 weeks (indicated by c_2 and c_3 in the figures). The addition of inosine is reflected by the increase in the levels of ribose, shown in Fig. 6. Samples were removed periodically from all the specimens for the estimation of ribose and the phosphate fractions. The results of the experiment are represented in Figs. 6 and 7.

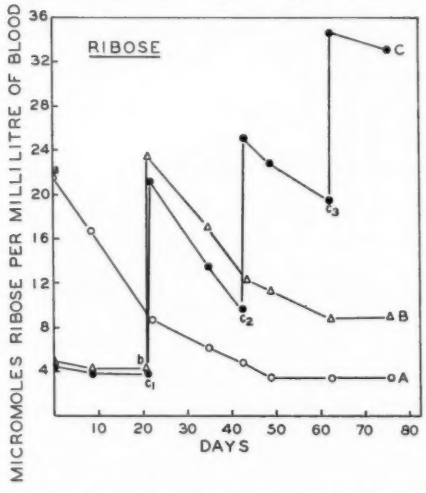

FIG. 6. Ribose utilization in blood specimens preserved in ACD medium at 4° C with repeated addition of inosine.
A. Inosine added at the beginning of storage.
B. Inosine added on the 21st day (*b*).
C. Inosine added on the 21st (c_1), 42nd (c_2), and 63rd (c_3) days.

The ribose (pentose) concentration in the control specimen A approached the minimum about the 50th day of storage. The concentration of P_I in this specimen increased slowly from the beginning of storage, while in specimens B and C, it rose more rapidly. Upon addition of inosine to the last two specimens at the end of the third week, the characteristic regeneration of organic phosphate esters (notably DPG) occurred, as reflected by the decrease in the P_I concentration. Resynthesis of ATP also occurred. The synthetic phase reached the maximum (minimum P_I concentration) about the 35th day of storage, and thereafter gave way to hydrolysis of the esters.

Upon the second addition of inosine (at c_2) to specimen C another phase of synthesis was induced, as indicated in Fig. 7. By the repeated addition of inosine in this manner, ATP can be maintained at a high level for a long time. It is evident that the synthetic phase induced by the addition of nucleoside was terminated when the supply of nucleoside approached depletion.

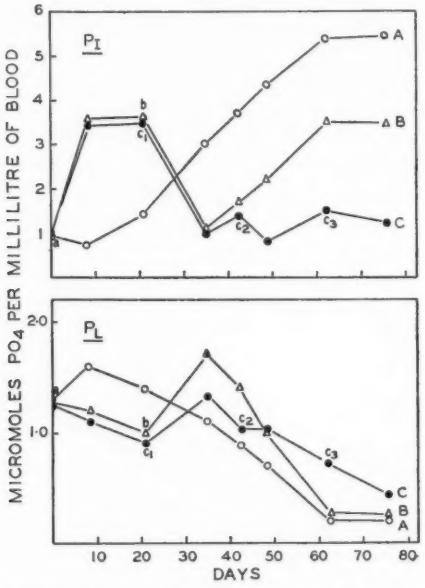

FIG. 7. Inverse behavior of inorganic and labile phosphate in blood on repeated addition of inosine during storage at 4° C.
A. Inosine added at the beginning of storage.
B. Inosine added on the 21st day (*b*).
C. Inosine added on the 21st (c_1), 42nd (c_2), and 63rd (c_3) days.

5. *The Rate of the Induced Ester Synthesis in Erythrocytes during Storage at 4° C*

To two portions of blood, which had been preserved in ACD medium at 4° C for 14 and 35 days, respectively, sterile inosine was added to give a final concentration of 800 mg%. The specimens were again stored at 4° C.

Periodically, during the following 55 hours samples were removed aseptically for estimation of the various phosphate fractions. The results are represented in Fig. 8.

The concentration of DPG and hexosephosphate in the red cells rapidly increased, and approached a constant value about the 30th hour. The concentration of ATP increased more slowly and became steady also about the 30th hour. The concentration of P_I, as usual, fell fairly rapidly at first, then more slowly, and reached a constant value about the 30th hour.

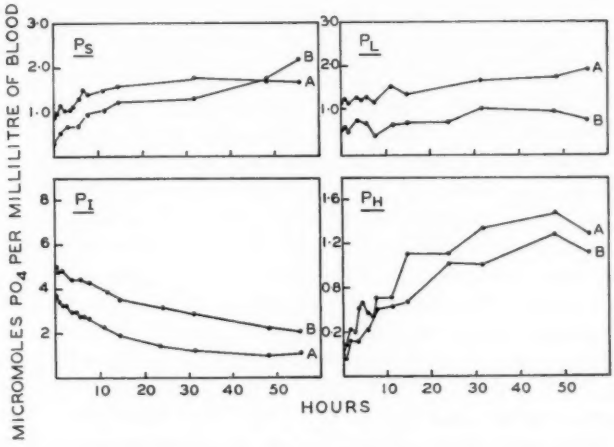

FIG. 8. Comparison of the influence of inosine on the behavior of the phosphate fractions in 14- and 35-day-old blood specimens stored at 4° C in ACD medium.
A. Specimen which had been stored at 4° C in ACD medium for 14 days before inosine was added.
B. Specimen which had been stored at 4° C in ACD medium for 35 days.

Discussion

The most important condition for the maintenance of viability in preserved erythrocytes is the maintenance of the ATP reserve of the cells. The level of ATP determines the capacity of the cells to maintain the synthetic activities of metabolism and to exclude sodium and retain potassium against steep gradients of these ions. ATP thus indirectly controls the volume and osmotic stability of the cells.

Among the conditions that tend to impair the capacity of the erythrocytes to replenish ATP during storage is an increase in the concentration of H-ions. This occurs progressively during storage in blood preserved in CD medium owing to the accumulation of lactic acid. In the ACD medium the H-ion concentration is reduced to about pH 7 at the outset by the acidic reaction of the medium (pH 5.5). Because of the pronounced sensitivity of hexokinase to H-ions, this enzyme (and consequently the glycolytic system) is progressively inhibited with increase in the H-ion concentration, and brought almost

to a standstill when the pH reaches 7. This occurs in CD-preserved specimens (at 4° C) about the end of the second week. At the latter pH there is an abrupt increase in the 2,3-diphosphoglycerate phosphatase (DPGase) activity which rises to a sharp maximum at pH 6.8 (1). The combined influence of the H-ion concentration (pH 7) on hexokinase and DPGase largely accounts for the inability of the cells to regenerate ATP and for the rapid increase in the concentration of inorganic phosphate at the end of the second week in blood preserved in CD, and from the beginning of storage in ACD at 4° C. It is noteworthy that while the rate of replenishment of ATP in blood preserved in ACD medium is greatly reduced, the rate of expenditure and hydrolysis of ATP also is greatly retarded at the low pH, thus resulting in a more prolonged conservation of the energy reserve.

The addition of inosine or other purine nucleoside to blood specimens after a period of storage (e.g. 3 weeks) in CD or ACD in the cold, effects a rapid reversal of most of the autolytic changes that have occurred during storage. Within a few hours, even at low temperature (4° C), the organic phosphate intermediates of the glycolytic system, notably ATP and DPG, are resynthesized to a high level despite low pH (pH 7) of the specimen. DPG is restored virtually to the original (normal) level even in very old (60-day) specimens whereas the degree of restoration of ATP becomes progressively less with increase in the storage age of the sample. It is of interest, as Brownstone and Blanchaer (3) have observed, that the activity of phosphofructokinase is increased to higher than the normal value on the addition of nucleoside (adenosine) to 20-day-old ACD-preserved blood specimens. The restoration of ATP and other esters is accompanied by a restoration of the electrolyte composition of the cells with a decrease in cell volume and improvement in the osmotic stability.

The remarkable action of the nucleoside in reversing the changes that occur during storage is attributable to the utilization of the ribose moiety of the nucleoside with replenishment of the ATP reserve. The pentose metabolic pathway, unlike the initial steps of the glycolytic route, is relatively unaffected by an increase in the hydrogen-ion concentration between pH 7.4 and 6.2. Thus, while the cells are unable to utilize glucose at pH 7 they can readily utilize ribose and do so without the expenditure of ATP. If the same cells be washed and resuspended in a glucose-containing medium of pH 7.4 or 8.0 they may be able to utilize glucose, but the rate of utilization always is diminished with increase in the age of the specimen, owing to the progressive and apparently irreversible impairment of hexokinase during the storage period.

The ability of the inosine-preserved cells to utilize glucose, on being washed and resuspended in a glucose-containing medium at pH 7.4, is best preserved in blood to which nucleoside is added at the beginning of the storage period. Thus, even though ribose is utilized almost exclusively by the red cells when the H-ion concentration approaches pH 7, the presence of nucleoside results in an increased preservation of hexokinase, possibly by virtue of the maintenance of ATP and a more normal cell composition. The ability of the

washed and resuspended preserved cells to utilize glucose becomes progressively diminished the later the time when inosine is added in the storage period.

Inosine or other purine nucleoside undergoes phosphorolysis at a fairly rapid rate in the presence of erythrocytes, and the period of induced synthetic activity continues only as long as ribose is available. Since addition of nucleoside to blood at the end of the second or third week gives practically as good results as addition at the beginning of storage, it is altogether likely that if inosine should be used in blood preservation its chief usefulness will be for the reclamation of 3-week or older preserved specimens. The nucleoside is stable to autoclaving in water or in ACD (pH 5.5). Gabrio and Finch (4) and workers in and associated with our group (5) have shown, by means of infusion of preserved red cells labelled with Cr^{51}, that the addition of inosine to 3-week-old blood specimens effects a significant improvement in the duration of cell survival in the circulation. This again supports the inference that the metabolic and biologic viability of the preserved cells is related to the state of the ATP reserve of the cells.

The main deterrent to the use of inosine for the preservation of blood for clinical use at the present time is the lack of information about the capacity of the human body to eliminate the uric acid that arises from the metabolism of inosine (hypoxanthine). A concentration of 500 mg% of inosine in a 500-ml unit of blood would yield about 1.5 g of uric acid. While this concentration of inosine appears to be well tolerated by a normal subject one would hesitate to administer the blood to a patient, in the present state of our knowledge. Inosine could be removed from the specimens by washing the cells, but this is too cumbersome a procedure to be practicable, and would result in waste of the plasma.

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THE EFFECT OF VAGOTOMY AND DENERVATION OF THE CAROTID SINUS ON DIURESIS FOLLOWING PLASMA VOLUME EXPANSION[1]

J. W. PEARCE[2,3]

Abstract

The hypothesis exists that afferent vagal fibers, arising in cardiac atrial stretch receptors, serve as the sensory limb of a reflex mechanism sensitive to variations in blood volume and producing corresponding changes in urine flow. Section of the vagi did not, however, prevent the diuretic and chloruretic response to expansion of the plasma volume with isotonic infusions. Carotid sinus denervation alone or combined with vagotomy also failed to prevent the diuresis and chloruresis following infusion in most experiments, although in a few cases the response was absent or diminished after the combined procedure. Reasons are given for believing that the bovine albumin infusion used did not act directly on the kidney to increase urine output, but rather through a reflex mechanism which could not have depended on the integrity of the vagi. It is concluded that receptors additional to those in the cardiac atria and the carotid sinus must contribute to the sensory component of a reflex mechanism regulating plasma volume.

Introduction

There is much evidence (1, 2) that changes in the volume of circulating blood, without alterations in plasma osmotic pressure, may result in compensating changes in urine flow. Infusions of plasma (3, 4), iso-oncotic albumin solution (5), and blood (6) have been shown to produce diuresis, and mild haemorrhage is well known to result in oliguria. Reduction of circulating blood volume by trapping blood in the extremities with tourniquets results in antidiuresis (7), and immersion of the body in warm water or exposure of the body to cold, procedures which probably increase the central blood volume, are followed by diuresis (8, 9). The prompt diuresis produced by the recumbent position in subjects previously infused with isotonic saline solution (10), and the diuresis produced by negative pressure breathing (11), suggest that an increase in thoracic blood volume acts as the stimulus to increased urine flow seen to follow expansion of the total blood volume. Gauer, Henry, Sieker, and Wendt (11) have offered the hypothesis 'that stretch receptors in the pulmonary circulation and/or chambers of the heart and great veins may represent the sensitive part of a mechanism linking haemostatic events with urine secretion for the control of plasma volume'.

The preceding hypothesis was strengthened by the finding in the dog that distension of a balloon enclosed in the left atrium resulted in striking diuresis with little change in systemic vascular pressures (12). Fibers from receptors

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sensitive to stretch of the atria have been demonstrated in the vagus of the cat (13) and of the dog (14). Such atrial stretch fibers show a marked increase in discharge during distension of the balloon in the left atrium, and moderate increases in activity during negative pressure breathing. The activity is also remarkably altered by small infusions or haemorrhages which have been shown to alter atrial pressures (15). It has been reasonably proposed that these fibers may act as the afferent limb of a reflex mechanism for the control of the volume of circulating blood. If this proposal be correct, vagotomy should abolish the diuretic response to plasma volume expansion.

In the work to be described, integrity of the vagus nerves did not prove essential to the diuresis which resulted from infusion of isotonic bovine albumin solution. The demonstration of stretch receptors in the carotid sinus (16) suggests the possibility that these endings may also be involved in regulating blood volume. The effect was therefore studied of denervation of the carotid sinuses, as well as section of the vagi, on the diuretic response to plasma volume expansion. In the hope of further elucidating the mechanism of the diuresis, urine chloride excretion was determined in most experiments.

Methods

Female dogs of medium size were used, anaesthetized with 90 mg/kg chloralose (1% solution in 0.6% saline) given intravenously following an earlier sedative injection of 1 mg/kg morphine sulphate. A maintenance dose of approximately 15 mg/kg of chloralose was given every 30 minutes, the quantity being adjusted to produce a depth of anaesthesia which still allowed brisk limb jerks in response to a loud noise. The animals were allowed to drink water up until the morning of the experiment, but had been fasted since the preceding afternoon.

The mean arterial pressure (BP) was recorded from a mercury manometer connected to saline-filled polyethylene tubing inserted into the aorta via the femoral artery. Right atrial pressure (RAP) was registered on a saline manometer connected to similar tubing inserted into the right atrium via the external jugular vein. Left atrial pressure (LAP) was recorded by a glass float in a saline manometer connected to a No. 9 Bard X-ray catheter which had been passed, with the aid of fluoroscopy, down the left carotid artery, into the left ventricle, and out into the left atrium. Occasionally an abnormally high LAP reading was recorded, presumably owing to mechanical interference with closure of the mitral valve, and the catheter was withdrawn. Heparinized saline was occasionally flushed through the tubings in order to delay clotting. Kymograph tracings were made of BP and LAP at intervals.

The carotid arteries were exposed through bilateral cervical incisions. The vagosympathetic trunks (hereafter referred to as 'vagi') were isolated and each was surrounded loosely with two ligatures. In preparation for carotid sinus denervation, the internal and external carotid arteries were carefully separated from surrounding tissue and retracted, the carotid sinus was lifted up, and two loose ligatures were passed around all remaining tissue emerging from the

sinus region. In almost all experiments a rise in BP resulted from section of each vagus nerve and section of each bundle containing the carotid sinus nerve; the left carotid sinus filled from above, although it was ligatured further down for insertion of the left atrial catheter.

Urine collections were made every 10 minutes by washing out the bladder with 20 ml of distilled water passed through a shaped glass urethral catheter perforated along its last 2 inches. Determinations of chloride content, by the Volhard–Henry method, were made on an aliquot of each washout sample. A correction was made to compensate for the 8-ml capacity of the collecting tube.

Infusions of Ringer–Locke solution and of 6% bovine albumin (Armour Laboratories) in Ringer–Locke solution were given into the femoral vein at 2 ml per kg per min and at 1 ml per kg per min, respectively. No reactions to the protein were apparent. The quantity of the infusion varied, and was usually approximately equivalent to 25% of the blood volume, estimated as 7% of body weight. Blood was withdrawn from the jugular or femoral vein.

Freezing point depression was determined for some samples of bovine albumin solution and of plasma of the recipient dog. A standard cooling apparatus was employed, using an ice and salt freezing mixture. The thermometer used was calibrated in hundredths of a degree centigrade, and the readings were corrected for zero error.

The general plan of the experiments was as follows. In all experiments the primary object was to determine the effect on urine flow and urine chloride content of infusions given before and again after section of nerves. In *series I* (control, six animals) no infusions were given but the effects of bilateral vagotomy were observed. In *series II* (14 animals) infusions were given before vagotomy and again after an interval of at least 30 minutes after nerve section. In *series IIIa* (six animals) infusions were given before and after combined vagotomy and carotid sinus denervation; *series IIIb* (four animals) had the same object, but infusions were given first after carotid sinus denervation and secondly after vagotomy performed later on the same animals. The latter series provided a few control observations of the effects of carotid sinus denervation alone, as there was a delay after the denervation before the first infusion began.

Results

Control Values (All Series) and Effect of Nerve Section Alone (Series I)

The vascular pressures were within normal ranges in the control period; the mean values were BP, 142 mm Hg; LAP, 6.1 cm saline; RAP, 3.9 cm saline. The control urine flow (V) varied between 2.1 and 14.6 ml/10 min, with an average of 4.6 ml/10 min. Urine chloride concentration (U_{Cl}) and total excretion ($U_{Cl}V$) varied widely, with average values of 84 meq/l. (range 14–188) and 0.4 meq/10 min (range .04–.95), respectively. Such a wide range of electrolyte excretion probably resulted from variations in depth of anaesthesia and from lack of uniform prehydration and dietary salt content;

the range of weights of dogs was 9.2–15.5 kg. Spontaneous diuresis occurred on several occasions at the beginning of the control period, and it was observed that the urine flow tended to vary inversely with the depth of anaesthesia.

The effect of bilateral vagotomy is typified by the results shown in Fig. 1B. The BP always rose on section of the nerves and usually returned to a level slightly higher than that of the control period; the LAP and RAP usually fell slightly. The urine flow often showed a transient reduction followed by a slight increase during the hour after vagotomy. A substantial diuresis never occurred. Total chloride excretion was little affected by the procedure, nor was there any consistent change in chloride concentration except when the transient antidiuresis was more pronounced. In these cases the chloride concentration rose sharply during one or two 10-minute collection periods and then returned to the range of control values.

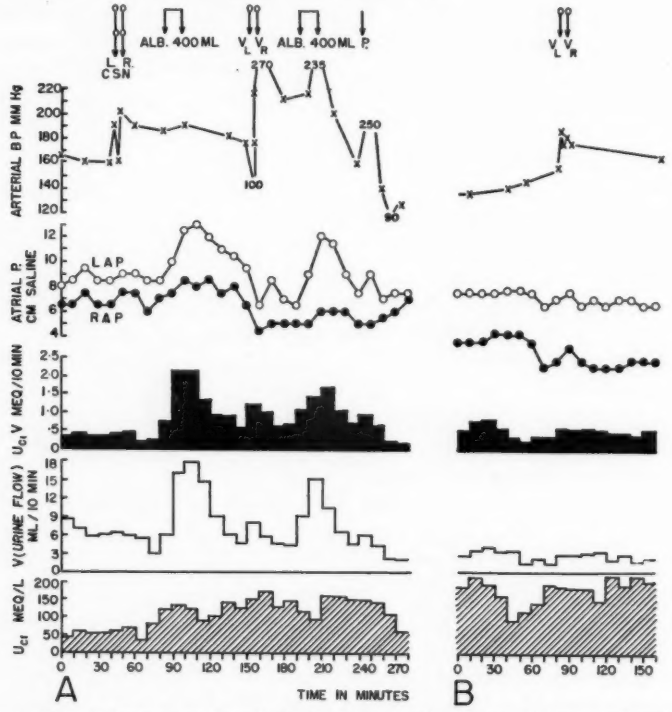

FIG. 1 (A). Effects on urine and chloride output of infusions of bovine albumin solution (ALB.) given first after carotid sinus denervation (CSN) and later after bilateral vagotomy (V_L V_R) in the same animal. RL, Ringer–Locke solution; ALB., 6% bovine albumin in Ringer–Locke solution; BP, arterial blood pressure; LAP, left atrial pressure; RAP, right, atrial pressure; $V_{Cl}V$; total chloride excretion (solid graph); V, urine flow (open graph); U_{Cl}, urine chloride concentration (hatched graph). P indicates an injection of pitressin, irrelevant to this report. Neither carotid sinus denervation alone nor the addition of bilateral vagotomy prevented the diuretic response to infusion.

(B). Effects of bilateral vagotomy alone on vascular pressures and on urine flow and chloride excretion. The symbols and scales at the left also apply here. The denervation had no significant effect on urine flow and chloride output.

Denervation of the carotid sinuses alone caused a rise in BP level but little effect on LAP and RAP. Urine flow and chloride content were not significantly altered. These features are seen in Fig. 1A.

Denervation of carotid sinuses in combination with bilateral vagotomy caused a more marked rise in BP, which was sustained. The pressure often rose above 200 mm Hg. This procedure usually reduced LAP and RAP and almost always caused antidiuresis and marked reduction in total chloride excretion. Changes in chloride concentration were variable. Some of these features are seen in Fig. 1A. In 3 of 13 experiments, complete anuria resulted from this drastic procedure.

At no time did any denervation procedure produce a significant increase in urine flow.

Series II. Effect of Infusates before and after Vagotomy

Infusions of both Ringer–Locke solution (RL) and 6% bovine albumin in Ringer–Locke solution (bov. alb.) almost uniformly increased the RAP and LAP; the increase in LAP was always the greater. The rise in BP was small or negligible.

The increase in urine flow was considered to represent a 'diuresis' if V was doubled during at least two 10-minute collection periods, or if it increased by 5 ml/10 min during two periods in cases of larger initial urine flow. Total chloride excretion was considered to have increased if $U_{Cl}V$ doubled during at least two collection periods.

The infusion of either RL or bov. alb. caused a diuresis in all but a few experiments, and usually increased the excretion of total chloride in the experiments in which it was measured. Chloride concentration showed a variable change after either infusion, but occasionally fell progressively during the diuresis; it seldom rose remarkably.

After vagotomy both RL and bov. alb. uniformly increased RAP and LAP and usually produced small increases in BP. Bov. alb. uniformly caused diuresis and saline failed to produce a diuresis only once in 11 experiments. Chloride concentration again was variably altered, but chloride excretion always rose. The postvagotomy diuresis was greater as often as it was smaller than that produced by a similar infusion before nerve section. These results are represented in Fig. 2, and summarized in Table I. Vagotomy did not, then, obviously alter the diuretic response to plasma volume expansion with RL or bov. alb. solutions.

Series IIIa and b. Effect of Infusates after Both Vagotomy and Carotid Sinus Denervation

In about half the experiments vagotomy and carotid sinus denervation were performed within one 10-minute period; in the other half there was an interval of 2 hours between the two procedures. This difference in design did not obviously alter the results, which are represented in Fig. 1A and summarized in Table I. As mentioned before, the combined denervation resulted in anuria in three dogs and these experiments were discontinued.

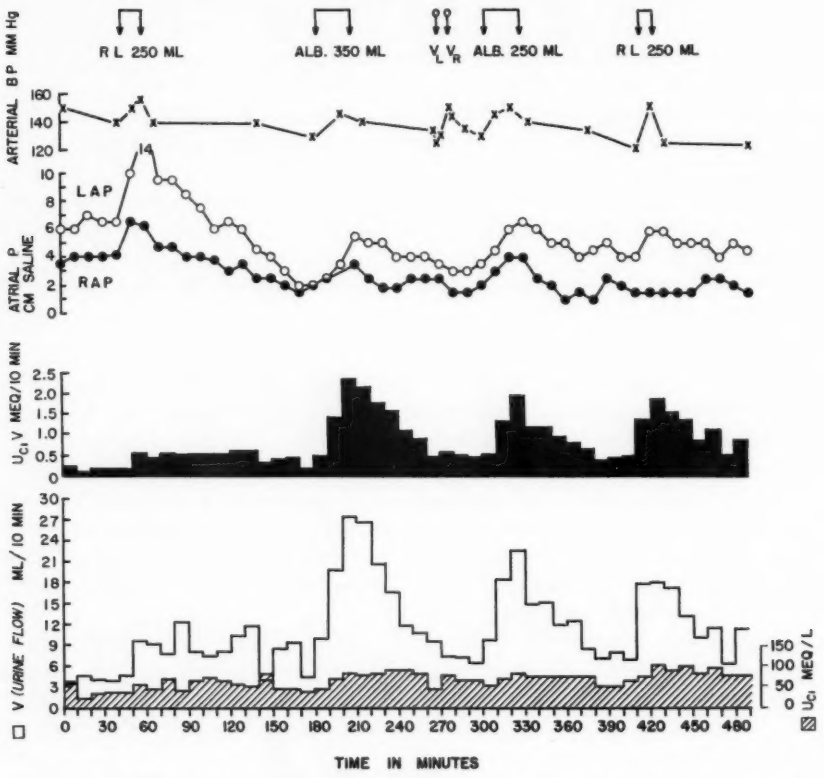

FIG. 2. Effects on vascular pressures and on urine flow and chloride excretion of infusions given before and after bilateral vagotomy (V_L V_R). The symbols and abbreviations are as described for Fig. 1. Vagotomy did not abolish the diuretic response to infusion.

Infusions of both RL and bov. alb. solutions almost uniformly increased RAP and LAP; BP usually rose, but occasionally fell slightly. A diuresis followed the infusion of RL in each of three cases, and both total chloride excretion and chloride concentration rose. These diureses were smaller than those which usually followed similar infusions of RL during the control period.

A diuresis followed the bov. alb. infusion in six dogs, but the infusion failed to produce a convincing diuresis in three other dogs which had shown a normal urine flow shortly after the double denervation. In one of the latter group of dogs, three separate 50-cc haemorrhages also had no effect on urinary output. When diuresis occurred, total chloride excretion and chloride concentration were also increased. When the diuresis failed to occur, there was no convincing chloruresis over the level seen earlier during a period when the animal was not showing diuresis.

Section of the vagi with carotid sinus denervation did not uniformly prevent the diuretic response to bov. alb. infusion. In a minority of experiments, however, the diuretic response was much reduced or prevented by the effects of the procedure.

Series IIIb. Effect of Infusate after Carotid Sinus Denervation Alone

In three animals bov. alb. infusion was given after the denervation of the carotid sinus regions (Fig. 1A). The resultant increase in RAP and LAP was similar to that seen in series II; the BP rise was also slight or negligible. A diuresis resulted in these cases, and the total chloride excretion was increased. Chloride concentration fell in two cases and rose in the third.

Carotid sinus denervation was not shown to interfere with the diuretic response to bov. alb. infusion.

TABLE I

Summary of results

Procedure	Experiment series	No. of experiments	Urine flow			Total chloride excretion		
			Diuresis	No effect	Antidiuresis	Increase	No effect	Decrease
Vagotomy alone	I	6	–	6	–	2	3	1
C.S.D. alone	III*b*	4	–	4	–	–	4	–
Vagotomy and C.S.D.	III*a*	10	–	1	9	–	1	9
RL infusion	II and III*a*	13	10	3	–	3*	1	–
Bov. alb.	II and III*a*	14	12	2	–	9*	4	–
Bov. alb. after vagotomy	II	8	8	–	–	6*	–	–
RL after vagotomy	II	11	10	1	–	3*	–	–
Bov. alb. after C.S.D.	III*b*	3	3	–	–	3	–	–
Bov. alb. after both vagotomy and C.S.D.	III*a* and *b*	10	7	3	–	8	2	–
RL after both vagotomy and C.S.D.	III*a* and *b*	4	2	2	–	2	2	–

NOTE: C.S.D., carotid sinus denervation; RL, Ringer–Locke infusion; bov. alb., infusion of 6% bovine albumin in Ringer–Locke solution; * indicates that urine chloride determinations were not done on all experiments.

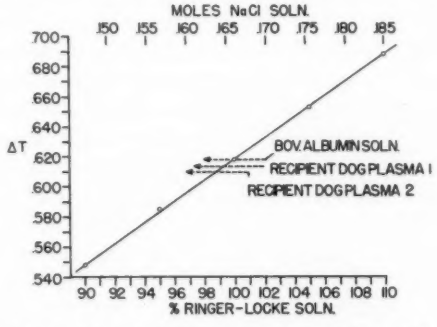

FIG. 3. A comparison of freezing point depressions (ΔT in °C, values indicated on ordinate by arrows) of 6% bovine albumin in Ringer–Locke solution and of the plasma of a dog subsequently infused with the solution. Plasma 1 was obtained during the control period and plasma 2 between the first and second infusion; a diuresis followed each infusion. The plotted points are determinations of ΔT for different concentrations of Ringer–Locke solution; osmotically equivalent concentrations of NaCl solution are given in the top scale.

Freezing Point Depression of Bovine Albumin Infusate and Plasma of Recipient Dog

In three experiments the freezing point depression (ΔT) of the plasma of the dog was compared with ΔT of the bovine albumin solution prepared for infusion. The results of such a comparison are shown in Fig. 3, in which the infusate was very slightly hyperosmotic to the plasma of the recipient dog; in the two other experiments the infusate was slightly hypo-osmotic. Each of the tested infusates produced a typical diuresis.

Discussion

The average control values for urine flow and chloride excretion were slightly higher than those usually found in the unanaesthetized dog without prehydration (17, 18). Variations in urine flow and chloride excretion during the control period were small enough to permit conclusions to be drawn regarding changes following the infusions; the fluctuations in chloride concentration during the control period, however, were seldom exceeded during the experimental periods. In a few experiments the diuresis in response to bovine albumin infusion was associated with a fall in chloride concentration, suggesting that reduced tubular reabsorption of water was a cause of the diuresis.

The effects of vagotomy and carotid sinus denervation alone were similar to those obtained by Malmejac and Galloni (19), who also used chloralosed dogs. They attributed the cessation of urine flow, which occasionally followed complete removal of the buffer nerves, to intense renal vasoconstriction; such was probably the explanation of the three occurrences of anuria reported here. The absence of any significant increase in urine flow or chloride content after carotid sinus denervation is in conflict with earlier work of Janssen (20, 21). He found that urinary water and chloride output rose following section of the carotid sinus nerve or clamping of the carotids. This diuresis was moderate and slow in onset (often after a 2-hour latent period) and was superimposed on a control urine flow of only 2 to 14 ml/hr. It is probable that if such a 'carotid sinus polyuria' occurred in the presently reported work, it was obscured by the much larger urine flows throughout the experiment.

The failure of vagotomy to abolish the diuretic response to iso-oncotic bovine albumin infusions would appear to contradict the suggestion of Henry, Gauer, and Reeves (12) that atrial stretch receptors serve as the sensory component of a reflex mechanism controlling blood volume. The contradiction may be resolved in two ways. Firstly, although it has been shown that cold block of the vagi *does* abolish or considerably attenuate the diuretic response to negative pressure breathing (1) and to distension of the left atrial balloon (14), these manoeuvres affect chiefly the thoracic viscera or left atrium. Infusions produce a generalized increase in vascular volume, however, and may affect other stretch receptors in the extrathoracic vascular system contributing to the sensory component of the reflex arc. Less probably, there may be pathways for atrial receptor fibres other than the vagus nerves. From the experiments reported here, the stretch receptors of the carotid sinuses

are not essential to the diuretic mechanism, and do not appear to play a major role in it. The attenuation of the diuresis and its occasional absence following section of both vagi and carotid sinus nerves may well have been due to the same phenomena leading to complete anuria in three of the experiments. To test the hypothesis that other pathways of atrial receptors are still active after vagotomy, it would be worth while to determine the effect of thoracic dorsal root section on the diuretic response to iso-oncotic infusions.

A second explanation for the contradiction of the hypothesis could be that the bovine albumin solution acted other than as a simple plasma volume expander in producing a diuresis. The occasional failure of this infusion to produce a diuresis argues against the likelihood that the infusate acted directly on the kidney, and suggests that the renal response is determined by a reflex mechanism sensitive to such variables as initial blood volume and anaesthetic depth. Excitation of the osmoreceptors, postulated by Verney (22), is an unlikely cause of the diuresis, as the freezing point depression of the bovine albumin solution used was shown, in several experiments, to be very close to that of the recipient dog plasma. Furthermore, considering the dilution of the hypo-osmotic infusions by the blood plasma, any reduction in plasma osmotic pressure would have been less than the minimum change, brought about by injections of NaCl solution, which was found by Verney to alter the secretion of antidiuretic hormone.

The possibility that the diuretic response to infusion resulted from an increased glomerular filtration rate is suggested by the associated increase in water and chloride output. Other work (5, 23) has shown, however, that the filtration rate is not increased by infusion of isotonic bovine albumin solution, even though increased sodium excretion does result (24, 23). The renal response to infusions of Ringer–Locke solution is not identical, however, as increased filtration has been shown to occur in the dog (25) although the mechanism is not clear. The bovine albumin infusate was probably nearly iso-oncotic to blood plasma (as opposed to the saline), which, associated with the fact that the arterial pressures changed little with the infusion, leaves no obvious cause for a direct effect on glomerular filtration. The possibility was not tested that bovine albumin escaped into the glomerular filtrate, but it seems unlikely that the osmotic diuretic effect of protein molecules of this size could have been responsible for the large diureses observed. The occasional decrease in urine chloride concentration implies that reduced tubular reabsorption of water contributed to the increased urine volume, consistent with other evidence (24, 5, 6) that inhibition of antidiuretic hormone secretion may result in the diuresis. It seems probable, therefore, that the diuretic response to plasma volume expansion, with bovine albumin solution at least, is determined by a reflex mechanism with extrarenal components.

The elevation in atrial pressures following the infusions was frequently maintained for over an hour. Such pressure measurements cannot be directly related to vascular volume but in the absence of infusion of vasoactive substances the persisting pressure elevation implies intravescular retention of

much of the infusate. This stimulus to cardiovascular stretch receptors still appears to have been the most likely factor initiating the renal response, but it is highly improbable that atrial receptors comprised the only sensory component involved.

Acknowledgments

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MECHANISMS OF THE RENAL RESPONSE TO PLASMA VOLUME EXPANSION[1]

E. L. ATKINS[2] AND J. W. PEARCE

Abstract

Expansion of the plasma volume by infusions either of plasma or iso-oncotic albumin solution resulted almost uniformly in diuresis and natriuresis in anaesthetized dogs. Although the clearance of para-aminohippurate (RPF) usually increased, changes in clearance of creatinine (GFR) were neither large nor consistent in direction, indicating that the renal response depended on altered tubular reabsorption. The usual temporal dissociation between the peak values for water and sodium output during the response was interpreted as evidence of operation of two effector mechanisms. Reasons are given for believing that the renal response was not due to a direct action of the infusate, but rather to activation of a reflex mechanism by expansion of vascular volume. Bilateral cervical vagotomy did not prevent either the diuresis or the natriuresis, again implying that receptors additional to those in the cardiac atria must contribute to the sensory limb of the reflex. The average magnitude of diuretic and natriuretic responses after cervical vagotomy was, however, less than a quarter of that before the denervation, supporting the contention that the vagi do contribute to the renal response to expansion of the plasma volume when plasma is infused. The diuresis and the natriuresis still occurred during infusion in adrenalectomized animals, indicating that inhibition of aldosterone secretion is not the only effector mechanism leading to a natriuretic response to expansion of vascular volume.

Introduction

Expansion of the central blood volume by a variety of manoeuvres has been shown to result in increased urine flow, sometimes accompanied by natriuresis, while reduction of blood volume may result in opposite changes (1, 2). There is a growing body of evidence, reviewed by Smith (2), that blood volume is regulated by a reflex mechanism producing compensating variations in renal output. Gauer and Henry (1, 3) have proposed that stretch receptors in the cardiac atria, with fibers in the vagus nerves, serve as the sensory component of the reflex, and electrophysiological recordings (4) have added evidence to support this hypothesis. In the previous paper (5), however, the vagi were shown not to be essential to the diuretic response to infusion of isosmotic bovine albumin solution. The diuresis in this case was associated with marked chloruresis, whereas increase in electrolyte excretion was an insignificant feature of diureses seen with negative pressure breathing (6) and during distension of a balloon in the left atrium (3); both of these diuretic responses could also be abolished or reduced by cold block of the vagi (3). In the light of these differences, it is possible that bovine albumin solution acted directly on the kidney of the dog rather than indirectly by expansion of the plasma volume. It was therefore considered essential to study the effect of vagotomy on the diuretic response in the dog to the infusion of canine plasma.

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The mechanism of the renal response to expansion of the total plasma volume is still undefined. There is, however, considerable evidence that the primary change is not in glomerular filtration rate, but rather in some parameter of tubular function (7, 8, 9, 10). The concept that inhibition of secretion of antidiuretic hormone (ADH) causes the diuresis is supported by the observation of Welt and Orloff (7) that administration of pitressin prevented the diuretic response to iso-oncotic infusions in human subjects. Related studies, involving sustained hydration (11, 12), isotonic infusions (10, 8), and mechanical redistribution of blood volume (13, 14), indicate clearly that renal tubular reabsorption of electrolytes and water are regulated independently during responses to expansion of the plasma volume. The infusion of plasma constitutes a more natural method of increasing the intravascular volume, but the renal response to this infusion has been infrequently or incompletely studied (15, 16, 9). A further investigation of renal function during infusions of plasma and isosmotic bovine albumin solution was therefore undertaken, with the secondary purpose of establishing that the mechanisms of diuretic response were the same with each infusion.

The mechanism of the natriuretic component of the response to expansion of the plasma volume is still unclear. Recent work by Bartter (17) and Davis (18) and their colleagues has implicated aldosterone as the principal steroid governing renal loss of sodium ion in states of expanded extracellular fluid volume where there is no change in osmotic pressure, and, paradoxically, in causing sodium retention after experimental production of increased venous blood volume. Hence it was thought worth while to determine the effect of adrenalectomy on the renal response to plasma infusion.

Methods

Preparation of the Experimental Animal

Mongrel dogs of both sexes weighing approximately 10 kg were used. No extra salt was provided and they were allowed water ad libitum until the morning of the experiment, but had been fasted since the preceding morning. Surgical anaesthesia was induced with an intravenous injection of sodium pentobarbital (30 mg/kg) and maintained by the addition of sodium pentobarbital to the constant infusion bottle. Polyethylene catheters were inserted into both femoral veins for infusion and blood withdrawal and into one femoral artery for registration of systemic arterial pressure by a mercury manometer. A saline manometer registered pressure in the right atrium from a catheter inserted via the right external jugular vein. Through a mid-line incision in the neck, both vagi were exposed in preparation for section later in the experiment. Both ureters were cannulated with fine polyethylene tubing through an incision in the left flank which allowed retroperitoneal exposure with a minimum of trauma. Urine was collected in 10-ml volumetric cylinders. The animal was minimally heparinized (1–2 mg/kg) and after a recovery period of ½ hour, the experimental procedures were begun.

The animals were infused either with plasma (series I) or with bovine albumin solution (series II) usually both before and after bilateral vagotomy. A small number of adrenalectomized animals was studied (series III) using the same techniques. Bilateral adrenalectomy was performed through a transverse abdominal incision and the animals were maintained for the following week before the experimental procedure on 25 mg of cortisone and 2.5 mg of desoxycorticosterone acetate given intramuscularly daily.

Sodium concentration (U_{Na}) in each urine sample was measured with a Beckman Model DU flame spectrophotometer; total sodium output ($U_{Na}V$) was calculated for each 10-minute period. The photometer calibration curve was prepared using artificial urine (19).

Plasma for infusion, obtained by slow exsanguination of donor dogs either at the time of the experiment or a few days previously, was centrifuged twice at 2500 r.p.m. for about 15 minutes; storage, where necessary, was in a refrigerator at 8° C. The plasma was filtered before use and infused into the animals at a rate of approximately 1 cc per kg per min, a rate found to have little effect on the blood pressure. Bovine albumin solution was prepared by mixing 6% by weight of the powder (Armour Laboratories) in warm Ringer–Locke solution; the infusate was filtered before use. The volume of either plasma expander was usually 18–24% of the estimated blood volume, calculated as being 7% of the animal's weight.

Renal Function Measurements and Preparation of Infusates

Exogenous creatinine and *p*-aminohippurate (PAH) clearances were measured using the constant infusion technique. An earlier paper (9) includes the formula used for calculation of the doses of these substances required to provide a plasma level of 7 to 15 mg% of creatinine and of 1 to 2 mg% of PAH.

Two consecutive 10-minute collections of urine, together with a venous blood sample at the end of the first collection period, were taken at important intervals throughout the experiment. Creatinine content in the plasma and urine samples was determined by the method of Bonsnes and Taussky (20) and PAH content was determined by a modification of the method used by Smith *et al.* (21) except that cadmium sulphate was used as the protein precipitant (22).

The renal clearance of creatinine was considered to be equivalent to the glomerular filtration rate (GFR) and the clearance of PAH to be equivalent to the effective renal plasma flow (RPF). Renal blood flow was calculated as $(RPF \times 100)/(100 - \text{venous blood haematocrit, \%})$.

Results

Data from Control Periods

The control period data in experiments with normal dogs gave average values of 3.5 ml/10 min for urine flow, 4.2 ml per kg per min for GFR, and 12.0 ml per kg per min for RPF. The ranges of apparently normal values for total sodium excretion ($U_{Na}V$) and for urinary sodium concentration (U_{Na}) were too wide to permit meaningful quantitative comparisons between animals.

No infusions were given to four "control animals" but frequent measurements of renal function were made before and after vagotomy. Vagotomy caused no apparent change in renal function values in the control animals, although a temporary increase in urine flow was observed in some of the experimental animals. The *average* variation about the mean value for each of the control dogs was determined to be $\pm 10\%$ for GFR and $\pm 11\%$ for RPF. This range of normal variation serves as a crude index of significance of experimental results (Fig. 4).

Series I

An infusion of canine plasma equal to at least 18% of the animal's estimated blood volume produced a diuresis in 13 of 14 dogs (Figs. 1 and 3A). A natriuresis occurred with each diuresis, and increase in U_{Na} usually reached its peak 10–30 minutes before the maximum water output and then fell during the period of maximum diuresis. Bilateral vagotomy did not prevent the diuretic and natriuretic response to a subsequent infusion, but the increase in $U_{Na}V$ was usually less and the U_{Na} tended to rise less and to remain more constant during the period of maximum water output. A few experiments were done (Table I) in which vagotomy was performed at the beginning of the experimental day to determine whether the first infusion influenced the response to a second infusion after vagotomy. There was no obvious difference between the patterns of the "postvagotomy" responses whether or not they

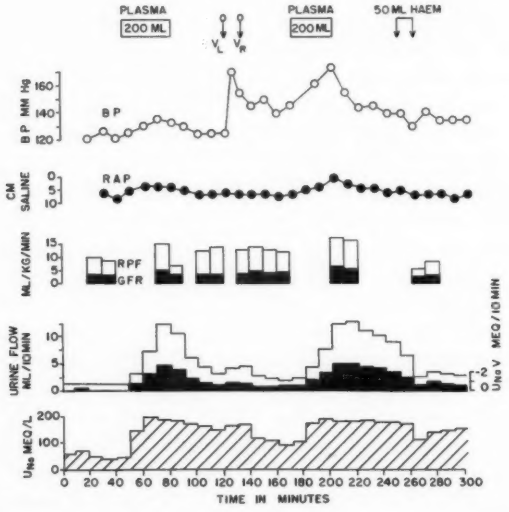

FIG. 1. The effects on renal function of infusion of canine plasma before and after vagotomy (V_LV_R). The effects of a small haemorrhage are also shown. BP, arterial blood pressure; RAP, right atrial pressure; RPF, effective renal plasma flow; GFR, glomerular filtration rate; urine flow, indicated by the open graph; $U_{Na}V$, total sodium excretion, solid graph (scale on right); U_{Na}, urinary sodium concentration, hatched graph.

were preceded by an earlier infusion, but the magnitude of the diureses was in both cases less than that seen with "prevagotomy" diureses. The average maximum increase in urine flow during the plasma infusion was 500% before vagotomy and 125% after vagotomy. The average maximum increase in $U_{Na}V$ was almost 600% before vagotomy and about 130% after vagotomy (Table I).

TABLE I

Effects on urine flow and urinary sodium excretion of infusions causing diuresis*

Dog	Urine flow (ml/10 min)				$U_{Na}V$ (meq/10 min)			
	Control (average)	Pre-vagotomy diuresis (peak)	Period immed. before 2nd infusion	Post-vagotomy diuresis (peak)	Period immed. before 1st infusion	Pre-vagotomy diuresis (peak)	Period immed. before 2nd infusion	Post-vagotomy diuresis (peak)
Series I (plasma infusion)								
A-18	2.6	7.7	4.2	8.0	.30	1.50	.75	1.20
A-19	1.3	22.0	4.2	5.5	.40	4.10	.45	.55
A-21	3.0	8.5	6.4	15.0	.20	1.20	.70	1.65
A-22	3.0	16.0	4.7	12.5	.19	1.85	.80	1.75
A-28	4.0	12.0	6.0	9.6	1.05	2.00	1.20	1.70
A-38	3.0	23.0	5.5	11.0	.50	3.50	.95	1.90
A-39	3.0	21.5	14.8	36.0	.05	.60	.65	1.90
A-41	5.0	20.5	4.9	12.0	.15	.25	.25	1.25
A-42	1.0	12.3	1.9	12.7	.05	2.60	.20	2.30
A-45	1.5	27.5	6.8	12.6	.02	2.30	.50	.90
Average	2.8	17.1	5.95	13.5	.30	2.00	.65	1.50
A-48	—	—	3.5	9.0	—	—	.60	1.25
A-49	—	—	4.0	3.5*	—	—	.45	.30*
A-50	—	—	2.0	6.3	—	—	.15	1.45
A-53	—	—	3.0	13.8	—	—	.40	1.70
Average			3.10	8.15			.40	1.20
Series II (bovine albumin infusion)								
B-24	2.0	8.8	3.7	11.6	.15	1.50	.75	2.30
B-26	6.5	28.2	7.7	14.2	1.35	5.10	1.60	2.95
B-28	3.0	4.5*	—	—	.37	1.04*	—	—
B-44	3.5	11.5	4.9	6.5	.65	1.70	.50	1.00
B-52	10.0	24.0	11.6	23.4	1.50	3.90	2.40	4.50
Average	5.0	15.5	6.9	13.9	.80	2.65	1.30	2.70
Series III (adrenalectomy and plasma infusion)								
D-25	2.5	17.8	3.7	2.0*	.45	3.25	.45	.10*
D-27	2.5	8.0	2.2	4.35	.60	1.10	.10	.30
D-29	2.2	8.1	1.0	4.4	.60	1.35	.01	.05
D-30	1.5	7.0	1.0	7.3	.25	.55	.05	.25
D-31	2.5	20.7	3.2	7.9	.05	.60	.15	.40
D-51	4.0	20.8	4.8	5.9	.50	.85	.02	.02
Average	2.5	13.7	2.5	5.30	.41	1.30	.13	.19

*Data are included from two experiments in which diuresis failed to occur.

There was no consistent alteration in the GFR values during the diureses when compared with control values. The GFR was almost as frequently observed to fall, or to remain unchanged, as to rise during the period of diuresis and natriuresis following an infusion (Fig. 4). The RPF values usually increased, however, during the diureses both before and after vagotomy (Table II). Such a finding might be expected as a result of the haemodilution accompanying infusion.

In nine experiments, small haemorrhages (50–100 ml) were effected during the recording period. Although the withdrawal was made slowly enough that no large fall in arterial pressure occurred, the urine flow fell promptly, associated with a temporary decrease in both GFR and RPF (Fig. 1). Values for $U_{Na}V$ usually fell proportionately and hence the U_{Na} did not change greatly.

TABLE II

Effects on renal function of infusions causing diuresis*

Dog	Glomerular filtration rate (ml per kg per min)				Effects on renal plasma flow (ml per kg per min)			
	Control	Pre-vagotomy diuresis	Post-vagotomy control	Post-vagotomy diuresis	Control	Pre-vagotomy diuresis	Post-vagotomy control	Post-vagotomy diuresis
				Series I (plasma infusion)				
A-18	5.55	5.30	—	—	10.2	8.75	—	—
A-19	3.20	3.40	3.75	3.45	6.1	9.2	7.35	8.0
A-21	4.55	5.50	4.80	5.50	11.3	16.2	13.0	11.5
A-22	4.10	6.20	4.55	5.90	11.8	15.95	13.0	15.0
A-28	5.00	6.55	5.60	5.00	14.75	19.3	18.7	16.8
A-38	3.70	3.75	3.80	3.75	10.0	15.35	11.6	13.8
A-39	—	—	4.05	3.80	—	—	11.5	10.7
A-41	4.1	2.95	3.15	2.70	15.4	12.6	11.0	12.35
A-42	2.65	3.55	4.0	5.35	9.0	10.5	11.95	16.55
A-45	3.90	2.80	—	—	10.15	15.75	—	—
Average	4.10	4.45	4.20	4.45	10.95	13.35	12.30	13.10
A-48	—	—	4.10	4.30	—	—	15.10	16.2
A-49	—	—	4.30	4.70*	—	—	13.8	16.6*
A-50	—	—	2.65	3.80	—	—	7.9	11.1
A-53	—	—	2.65	4.00	—	—	7.95	13.4
Average			3.60	4.20			11.15	14.30
				Series II (bovine albumin infusion)				
B-24	6.20	5.10	6.30	6.80	22.5	14.7	17.8	18.2
B-26	4.65	4.80	4.10	4.90	12.2	12.5	9.65	13.0
B-28	5.00	5.90*	—	—	14.75	18.0*	—	—
B-44	2.70	1.70	3.0	2.95	8.1	2.8	8.5	12.5
B-52	4.10	4.25	4.90	5.20	13.2	13.55	16.2	19.5
Average	4.55	4.35	4.60	4.95	14.2	12.3	13.05	15.8
				Series III (adrenalectomy and plasma infusion)				
D-25	5.80	5.35	3.85	6.1*	13.4	15.9	10.9	17.3*
D-27	5.3	5.35	4.9	5.1	12.2	18.55	16.8	20.8

*Data are included from two experiments in which diuresis failed to occur.

Series II

Five animals were given infusions of 6% bovine albumin in Ringer–Locke solution. No qualitative differences were detectable in four of these animals in the diuresis, natriuresis, or the renal function measurements when compared with the responses in other animals to plasma infusion (Fig. 2A, Tables I and II). In a fifth animal (B-28, Fig. 2B) a diuresis failed to occur, but an interesting observation was the progressive rise in U_{Na}; a diuretic response occurred later in this animal (A-28) following an infusion of plasma.

Haemodynamic Data (Series I and II)

The infusion of either plasma or bovine albumin solution caused inconsistent small alterations in both right atrial and systemic arterial pressures. Occasionally there was a slight fall in BP, or no alteration in BP or RAP (Fig. 3A), in spite of the production of a typical diuresis. When the RAP did rise, the peak usually occurred before the period of maximum urine output (Fig. 2A).

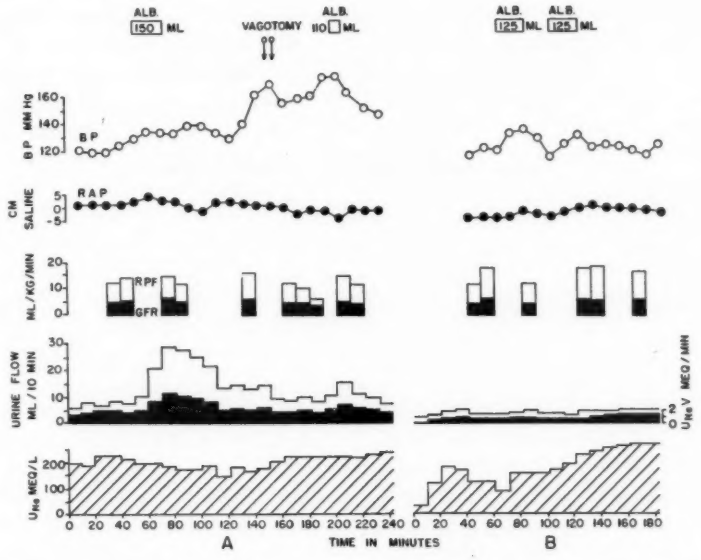

FIG. 2(A). The effects on renal function of infusion of 6% bovine albumin in Ringer–Locke solution, administered before and after vagotomy. The symbols and abbreviations are as for Fig. 1. The urinary sodium concentration falls during the first diuresis. The second diuresis is minimal, but the volume infused is also less.

(B). The renal response to two consecutive infusions of bovine albumin solution was atypical; the urine flow did not increase but the urine sodium concentration rose progressively. The designations and scales of Fig. 2A also apply here.

Series III

Six adrenalectomized animals withstood the experimental procedures and were infused with plasma both before and after vagotomy; three others showed a progressive fall in BP during the lengthy experiment and the results from these were discarded. A small diuresis resulted from every infusion but one, and in each case the $U_{Na}V$ also rose (Table I, Fig. 3B). The fall in U_{Na} was pronounced during the peak of the diuretic response. The renal function values were acceptable in only two experiments (owing to technical difficulties including excessively high plasma hippurate levels) but these showed no apparent difference from those recorded for the two previous series.

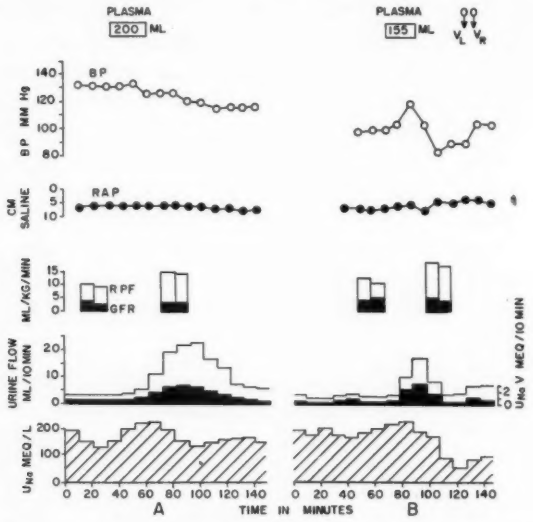

FIG. 3(A). The renal response to infusion of canine plasma is shown; note the increase in urinary sodium concentration coincident with the infusion, and the subsequent fall during the period of maximum diuresis. The symbols and abbreviations are as for Fig. 1.

(B). The renal response to infusion of canine plasma in an adrenalectomized dog is shown. The increase in total sodium excretion is as pronounced as that seen in Fig. 3A, although of shorter duration.

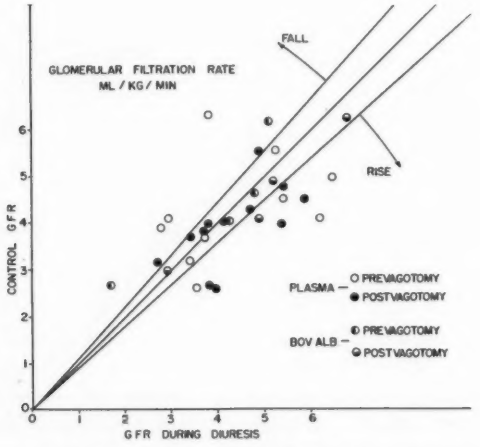

FIG. 4. Each point is a plot of the value for GFR during the control period against the value for GFR during the subsequent diuresis. The nature of the infusate, and the relation of infusion to the time of vagotomy, is indicated by the key. The lines on either side of the mid-line of no change enclose an area within which points could be expected to fall as a result of normal variation in GFR; these lines were derived from data obtained during control experiments. There was no consistent change in GFR during the diureses following either plasma or bovine albumin infusions.

Discussion

The observation that diuresis usually follows a moderate infusion of plasma confirms previous findings in the dog (15, 23, 9, 24) and in man (16). This occurrence of diuresis without any large or uniform change in GFR is consistent with earlier results of Young, Pearce, and Stevenson (9) and Wilson and Harrison (16), and again implies that a tubular mechanism is responsible for the increased water output. Of some interest is the associated natriuresis, as increased sodium output does not invariably follow manoeuvres designed to increase thoracic blood volume, although antinatriuresis is a consistent result of reduction of central blood volume (2). The natriuresis was also most likely to have resulted from altered tubular reabsorption, even bearing in mind Selkurt's view (25) that "small changes in GFR, undetectable by present techniques, may account for changes in $U_{Na}V$ often considered significant" in acute experiments. In many instances the GFR and $U_{Na}V$ changed in opposite directions, and in one animal (Fig. 3B) $U_{Na}V$ increased without change in water output. If it is accepted that the renal responses were independent of the filtration rate, the observation that increase in $U_{Na}V$ often preceded increase in V provides further indication that two mechanisms operated to alter tubular function. Inhibition of ADH secretion has been proposed to explain the diuretic response, and the usual decrease in U_{Na} at the peak of the diuresis is consistent with this concept.

The suggested effector mechanism for natriuretic response to increased extracellular fluid volume, which has received most recent support (17, 18, 26), is inhibition of aldosterone secretion. There is good evidence that, on the other hand, reduction of total extracellular fluid volume (17), as well as acute reduction of blood volume (27), increases the urinary excretion of aldosterone. The failure of adrenalectomy to prevent the natriuretic response to plasma infusion argues against the concept that regulation of blood aldosterone level is the *only* effector mechanism governing urinary sodium output during plasma volume expansion. Appropriate variations in $U_{Na}V$ during postural changes have also been clearly observed in patients with adrenal cortical insufficiency (28, 29). A rapidly acting natriuretic hormone or the activity of nerves to renal tubular cells may be speculated upon as additional or alternate mechanisms to the control of aldosterone secretion. Cort (30) and Keeler (31) have both demonstrated that hypothalamic lesions may cause altered urinary sodium output, and proposed that direct nervous control of tubular reabsorption exists. In view of some recent evidence (32, 33, 34) it is possible to explain these results as due to interference with a diencephalic center responsible for exerting hormonal control over adrenal cortical production of aldosterone. The evidence for existence of nervous control of tubular function is thus incomplete, and search for a natriuretic hormone, not associated with the adrenal glands, also seems warranted.

The possibility exists that substances in the separated plasma may have caused the diuresis rather than a mechanism dependent on the expansion of plasma volume alone. Histamine reactions are produced with the intracutaneous and intravenous injection of plasma from one dog into another (35),

and two animals, not included in our series, did suffer a precipitous drop in arterial pressure on plasma infusion which may have been caused by a histamine-like substance. The reported effects of small doses of histamine, however, include reduction of creatinine (36) and PAH (37) clearances in the human subject, and reduction in urine volume (38) and venous outflow from the perfused kidney of the dog (39). Although decreases in GFR and RPF were occasionally observed in the experiments reported here, these changes bore no uniform relationship to the diuresis and natriuresis. It seems unlikely, therefore, that histamine was the cause of the diuretic response, although varying levels of it may well have been responsible for the variability of renal function values during infusions. Epinephrine and norepinephrine could also have existed in the plasma used, since it was obtained by exsanguination of the donor animal. As the infusions never produced large rises in arterial pressure, the presence of even moderate amounts of such vasoactive substances in the plasma seems to be ruled out. Kaplan, Fomon, and Rapoport (40), using anaesthetized dogs, have shown that infusions containing 0.07–1.4 μg per kg per min of epinephrine consistently caused antidiuresis, with reduction of GFR and RPF, while similar doses of norepinephrine gave similar but less powerful effects. A dosage range of 3 to 10 μg/min in the human subject, which produced only small changes in mean blood pressure, brought about a fall in RPF and a fall in GFR at the larger dosage levels (41). Another possible constituent of the infused plasma, serotonin, produces a striking antidiuresis due largely to decreased GFR (42, 43). A final argument against the likelihood that substances in the infused plasma were responsible for the diuretic effects is the similarity of the response to that following infusions of bovine albumin.

The absence of consistent alterations in GFR following infusions of bovine albumin solution removes the uncertainty expressed earlier (5) that the infusions did not act to increase filtration rate. The unequivocal natriuresis produced by both this infusate and by plasma indicates that the response to over-all expansion of the plasma volume is an increased excretion of water *and* electrolytes. This contradicts the interpretation (2) placed on the results of Welt and Orloff (7) that infusion of iso-oncotic solution does not cause natriuresis. The results of their experiments, on human subjects, did demonstrate an increase in sodium output following infusion of iso-oncotic albumin solution, however, but it was not larger than that following infusions of similar amounts of saline. As saline infusions do not cause increased filtration rate in man (8), the results of Welt and Orloff might be reinterpreted to support the view that the diuretic responses to either isotonic saline or iso-oncotic solutions depend on the same mechanisms triggered by expansion of plasma volume.

The diureses following both plasma and bovine albumin infusions were usually associated with increases in RPF and a consequent reduction in calculated filtration fraction. The rise in RPF could be expected as a result of haemodilution; the calculated RBF tended to follow the RPF, however,

showing a rise more often than a fall. A constant or a reduced RBF, in the face of a much increased cardiac output and constant blood pressure, was shown earlier to follow more severe hypervolaemia from infusions of plasma (9) and was attributed to selective maintenance of renal vessel caliber in spite of general vasodilatation. The occurrence of diureses in both groups of experiments, however, without consistent change in GFR, contributes to the growing body of evidence that renal haemodynamic factors are not responsible for diuresis following plasma volume expansion.

Cervical vagotomy failed to prevent the water and salt diuresis following the infusion of plasma, although the response was almost always reduced in magnitude after the denervation. The sodium response to infusion was altered during the postvagotomy period in many cases; the alteration consisted of a less pronounced initial rise and subsequent fall in U_{Na} during the diuresis, although $U_{Na}V$ still increased. These differences were not considered striking enough to permit any conclusions about the effect of vagotomy on the sodium response, but suggest interference with the diuretic mechanism. If the only afferent pathway for the reflex regulation of blood volume lies in the vagi, as maintained in the hypothesis outlined earlier, vagotomy itself would have resulted in antidiuresis and should have prevented the response to infusion. Since neither of these results was the case, it seems necessary to conclude that atrial receptors play only a partial role in detection of acute alterations in plasma volume, and that other receptors must share this responsibility. The observations that cold block of the vagi prevented the diuretic response to negative pressure breathing and to atrial balloon distension (3, 4) are reconciled with this conclusion if atrial stretch receptors serve as the *thoracic* detectors of increased circulating blood volume while other receptors, existing outside the thorax, serve to register increased blood volume in other parts of the body. It is interesting to note that increase in $U_{Na}V$ is insignificant or absent during the diuretic response to NPB and balloon distension, whereas it is a prominent feature of the response to plasma volume expansion. The first two manoeuvres would stimulate only the thoracic receptors whereas infusion would act upon all receptors.

The site of extrathoracic receptors might logically be sought in the abdominal venous system, as congestion of the peripheral veins produces antidiuresis rather than diuresis, and venous congestion in the head has been shown by Epstein (44) to have no appreciable effect on urine flow or sodium content. The extensive observations that chronic congestion of the abdominal veins, caused by constriction of the inferior *vena cava*, produce water and salt retention are difficult to reconcile with this suggestion unless refuge is taken in the fact that the state of the animal, including perfusion of the liver, is quite abnormal. "Volume receptors" in the arterial tree have also been proposed by Epstein (44). An additional location for extracardiac receptors, as proposed by Bartter *et al.* (17), is the interstitial space. Particularly pertinent is the recent observation of Fine, Meiselas, and Auerbach (27) that the elevated aldosterone level resulting from haemorrhage is not corrected by prompt restoration of plasma volume, at the expense of interstitial fluid,

achieved by infusion of hyperoncotic albumin. Such an extravascular location of receptors contributing to the regulation of body fluid content and distribution would explain also the failure of hyperoncotic infusions to cause diuresis, in spite of increased plasma volume (7).

Acknowledgments

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THE EFFECT OF DAMAGE TO VARIOUS PARTS OF THE RENAL TUBULE ON THE EXCRETION OF PHOSPHATE BY THE DOG'S KIDNEY[1]

T. F. Nicholson and G. W. Shepherd

Abstract

The first third of the proximal tubules of the left kidney in dogs was damaged by the injection of 2.5 mg% potassium dichromate, the last two thirds by the injection of 0.5% sodium tartrate, and the distal tubules by the retrograde injection up the ureter of 0.05% mercuric chloride. Damage to the first third of the proximal tubule resulted in marked increase in phosphate excretion. Damage to the last two thirds of the proximal tubule had no significant effect on the output of phosphate. When the distal tubule was damaged the excretion of phosphate was greatly reduced. The results indicate that phosphate is reabsorbed in the first third of the proximal tubule and actively excreted by the distal tubule.

Introduction

In spite of marked differences of opinion on the quantitative mechanisms of tubular reabsorption of phosphate by the mammalian kidney, some investigators claiming that the tubules exhibit a maximal reabsorptive capacity, constant (1, 2, 3), or varying from time to time (4, 5), while others believe that they reabsorb a constant proportion of the filtered load (6, 7), it is still generally assumed that the tubules handle phosphate by reabsorption exclusively (5, 8). On the other hand tubular excretion of phosphate by some of the lower marine vertebrates (9, 10, 11) and in the chicken (12) has been clearly demonstrated. There have been suggestions that tubular excretion of phosphate by the mammalian kidney may occur (13, 14, 15), or that the possibility cannot be excluded (16, 17), but these suggestions have been ignored or rejected by most investigators. The experiments reported here were undertaken to obtain more definite evidence as to the role of the tubules in handling phosphate and to localize the sites at which phosphate transport occurs.

Experimental

Mongrel female dogs, weighing from 8 to 16 kg, fed on the stock laboratory diet and with water ad libitum, were used. The first third (approximately) of the proximal tubule was damaged by injecting 50 ml of 2.5 mg/100 ml potassium dichromate into the left renal artery and removing it from the left renal vein by the method previously used for sodium tartrate (18), and the last two thirds (approximately) of the proximal tubule was damaged by the injection of 0.5% *dl*-sodium tartrate in the same manner. The distal tubule was damaged by the retrograde injection up the ureter of 0.05% mercuric chloride, or in a few instances of 0.1% *dl*-sodium tartrate, by the method described by Nicholson and Downs (19). Seventy-two hours after the injection the animals

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were anaesthetized with sodium pentobarbital and the ureters were catheterized. A priming dose of 25 ml of 0.9% saline containing 0.5 g creatinine was given rapidly, followed by a sustaining dose of 0.25% creatinine in 0.9% saline at the rate of 0.75 ml per minute. After a stabilizing period of 30 minutes urine was collected from each ureter for two half-hour periods. Blood was taken from the femoral vein at the beginning, the middle, and the end of each period. It was heparinized and centrifuged immediately, and the plasma was removed for analysis. In a few experiments an osmotic diuresis was produced by administering a sustaining dose consisting of 0.25% creatinine, 10% mannitol, and 0.9% saline at the rate of 1 ml per minute. Inorganic phosphate was determined by Gomori's method (20) and the rates of excretion were calculated in micrograms per minute. Creatinine was determined by the method of Bonsnes and Tausky (21) and the clearances were estimated for each kidney. In a number of experiments, which will be reported in detail in a subsequent communication, the sodium and potassium in the urine were determined by flame photometry, urine glucose was estimated by the method described by Harding and Selby (22), and ammonia by extraction with permutit and nesslerization (23).

At the end of the experiment the kidneys were removed from the anaesthetized animals and fixed and examined by the methods previously described (18). Extra pieces were fixed in formalin for microdissection by the method devised by Oliver (24).

Results

The histological changes resulting from the injection of *dl*-sodium tartrate and mercuric chloride have been described in earlier communications (18, 19). The histological changes produced by potassium dichromate were quite similar to those produced by sodium tartrate but the distribution appeared somewhat different. On microdissection and staining with iron haemotoxylin by Oliver's method (24), the lesions produced by dichromate were found to be confined to the first part (roughly the first third) and the lesions produced by tartrate to approximately the last two thirds of the proximal tubule. This distribution corresponds fairly well with that shown by Oliver (24) for the lesions produced by dichromate and tartrate injected systemically. The lesions produced by the ureteral injection of mercuric chloride or *dl*-sodium tartrate extended through the collecting tubules and distal convoluted tubules as far as, but not into, the loop of Henle.

Table I lists the changes in the excretion of sodium, chloride, potassium, ammonia, and glucose produced by damage to the three different parts of the tubule. The excretion of both sodium and chloride is increased by damage to all parts of the tubule. The excretion of potassium is increased by damage to both portions of the proximal tubule and decreased slightly by distal damage. Ammonia excretion is not affected by dichromate or tartrate nephrosis but is sharply depressed in distal nephrosis. The excretion of glucose is significantly increased in dichromate nephrosis, slightly increased in tartrate nephrosis, and unaffected by distal tubular damage.

TABLE I

The effect of damage to different parts of the renal tubule on the excretion of various substances

	Dichromate nephrosis		Tartrate nephrosis		Distal nephrosis	
	Norm.	Neph.	Norm.	Neph.	Norm.	Neph.
Sodium*	(5) 25	58	(6) 36	69	(6) 26	69
Potassium*	(5) 9	20	(6) 15	19	(6) 15	10
Chloride*	(5) 28	63	(6) 42	76	(6) 20	53
Ammonia*	(2) 18	21	(4) 20	19	(2) 29	4
Glucose†	(4) 9.1	256	(6) 9.8	56.2	(4) 8.3	6.0

*Excretion in microequivalents per minute.
†Concentration as milligrams per 100 milliliters.
NOTE: The results are the average of several observations. The figures in parentheses represent the number of experiments involved.

The effects of damage to the first third of the proximal tubule (dichromate nephrosis) on phosphate excretion and creatinine clearance are shown in Table II. In Table III are shown the effects of tartrate nephrosis (affecting the last two thirds of the proximal tubule), and in Table IV, the effects of distal tubular damage.

The effects on phosphate excretion are shown both as gross percentage change and 'corrected' for the effects due to increased tubular permeability. As it has been shown that reductions in creatinine clearance resulting from degenerative tubular lesions are due to back diffusion through the nephrotic tubules (18, 25) and as it is probable that phosphate also diffuses back through the damaged epithelium, it is necessary, in determining the effects of the renal

TABLE II

The effect of damage to the first third of the proximal tubule (dichromate nephrosis) on the excretion of inorganic phosphate

Urine volume		Creatinine clearance			Phosphate excretion					
					μg/min					
Norm.	Neph.	Norm.	Neph.	'Correction ratio', nephrotic/normal	Norm.	Neph.	% increase due to nephrosis (uncorrected)	Normal excretion × 'correction ratio'	% increase using 'corrected normal'	Serum inorganic phosphate
0.09	0.1	13	13	1	145	228	57	145	57	3.6
0.18	0.47	42	42	1	304	528	74	304	74	4.1
0.75	1.95	47	46	1	192	357	86	192	86	5.0
0.16	0.37	40	37	0.9	139	200	44	125	60	4.3
0.12	0.21	34	35	1	99	240	142	99	142	4.8
0.11	0.14	10	11	1.1	151	315	109	166	90	4.2
0.36	0.71	29	28	1	52	90	73	52	73	3.7
0.22	0.56	26	27	1	48	100	108	48	108	4.2
0.25	0.39	22	23	1	61	106	74	61	74	4.5
0.14	0.18	32	29	0.9	150	393	162	58	191	3.9
		Average		0.99			92.9		95.5	

Normal dog						
Right	Left	Right	Left	Left/right	Right	Left
0.41	0.39	39	41	1.05	263	257

lesions on tubular activity, to estimate the changes in excretion over and above those due to increased back diffusion. The gross percentage change in phosphate excretion is calculated using the actual difference in phosphate output between the normal and damaged kidneys in the same animal. The 'corrected' percentage change is estimated using the difference between the actual phosphate excretion by the nephrotic kidney and that which might be expected if the only effect of the lesion were increased permeability. This latter amount is estimated by multiplying the figure for phosphate excretion from the normal kidney by the ratio of the creatinine clearance by the nephrotic kidney to the creatinine clearance by the normal kidney. This correction is based on the assumption that the glomerular filtration rate is the same for the normal and nephrotic kidneys in the same animal. This can not be proved definitely but as there is no histological evidence of glomerular damage, as the

TABLE III

The effect of damage to the last two thirds of the proximal tubule (tartrate nephrosis) on the excretion of phosphate

Urine volume		Creatinine clearance			Phosphate excretion					
					μg/min		% change due to nephrosis (uncorrected)	Normal excretion × 'correction ratio'	% change using 'corrected normal'	Serum inorganic phosphate
Norm.	Neph.	Norm.	Neph.	'Correction ratio', nephrotic/normal	Norm.	Neph.	+ −		+ −	
0.76	0.32	22	8	0.36	474	158	67	171	8	5.3
0.36	1.0	43	17	0.40	822	307	63	329	7	5.1
0.45	0.68	32	21	0.66	496	295	41	327	10	4.5
0.31	0.57	40	23	0.58	61	56	8	35	60	4.1
0.27	0.42	32	15	0.47	121	62	49	57	9	3.9
0.13	0.25	30	15	0.50	205	78	62	103	24	4.3
0.24	0.29	24	19	0.79	115	59	49	91	35	4.1
0.19	0.26	22	17	0.77	19	16	16	15	7	4.6
0.88	1.20	54	25	0.46	25	10	60	12	17	3.7
	Average						0 46		25 17	

TABLE IV

The effect of distal tubular nephrosis on the excretion of inorganic phosphate

Urine volume		Creatinine clearance			Phosphate excretion					
					μg/min		% decrease due to nephrosis	Normal excretion × 'correction ratio'	% decrease using 'corrected normal'	Serum inorganic phosphate
Norm.	Neph.	Norm.	Neph.	'Correction ratio', nephrotic/normal	Norm.	Neph.				
1.3	1.56	23	13	0.56	498	51	90	279	82	4.4
0.62	0.43	52	30	0.58	171	14	92	99	86	4.8
0.16	0.81	52	32	0.62	77	18	77	48	63	5.3
0.47	0.48	38	28	0.74	498	122	76	368	67	4.2
0.25	0.97	38	22	0.58	328	76	77	190	60	4.2
0.11	0.18	29	21	0.72	222	83	63	160	48	3.4
0.12	0.32	27	21	0.78	250	65	74	195	67	4.6
0.12	0.24	43	31	0.72	283	105	63	204	48	4.3
0.19	0.57	48	29	0.60	350	16	95	210	92	5.0
0.45	0.65	R 32	25	0.78	210	40	81	164	76	5.0
0.65	0.88	R 27	21	0.78	362	71	80	282	75	3.5
0.16	0.38	R 46	33	0.72	215	81	62	155	48	4.9
		Average					77		68	

blood flow, determined on a limited number of animals by direct observation of the rate of flow of renal vein blood through a calibrated glass loop, was essentially the same in the normal and nephrotic kidneys in the same animal (26), and as during osmotic diuresis the nephrotic/normal creatinine clearance ratio approaches unity (Fig. 1), it is probable that the glomerular filtration rate is unaffected by the nephrosis. It is obvious that such a correction can be approximate only, as it is unlikely that the back diffusion of phosphate is exactly the same as that of creatinine, but the 'corrected' figures should represent a fairly close approximation to the actual effects of the tubular damage on phosphate excretion.

Damage to the first third of the proximal tubule has little effect on the excretion of creatinine. The effect on phosphate excretion, however, is striking. There is in all experiments a definite increase, varying from 57% to 191%, with an average increase of 95% in the phosphate excreted by the damaged kidney.

The kidneys with tartrate nephrosis (the last two thirds of the proximal tubule) had creatinine clearances of from 36% to 79% of the normal kidney in the same animal. The excretion of phosphate from these kidneys showed no definite pattern of change and in most instances the deviation from normal, other than that which could be accounted for by the back diffusion due to tubular damage, was not significant.

With distal tubular damage the creatinine clearances varied from 56% to 78% of normal. In every experiment the excretion of phosphate from the damaged kidney was less than that from the normal kidney in the same animal. The 'corrected' decrease varied from 48% to 92% with an average decrease of 68%.

The effects of osmotic diuresis on creatinine clearance and phosphate excretion were observed in four animals with tartrate nephrosis and four with distal nephrosis. In all cases the nephrotic/normal creatinine clearance ratios, which varied from 0.45 to 0.66 before the onset of the diuresis rose with the increasing urine volume. When the rate of flow from the nephrotic kidney reached 5 ml per minute the clearance ratios varied from 0.93 to 0.97. The creatinine clearance by the normal kidney showed no significant change with variations in urine volume.

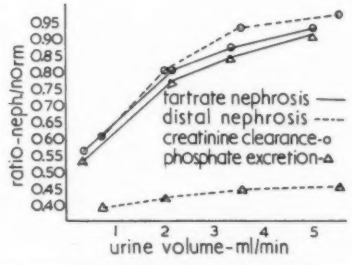

FIG. 1. The effect of osmotic diuresis on the creatinine clearance and phosphate excretion in tartrate nephrosis and in distal nephrosis.

The effects of the diuresis on the nephrotic/normal phosphate excretion ratio paralleled those on the creatinine clearance ratios in the animals with the tartrate nephrosis, but in those with distal nephrosis the change in the ratio was very small. Typical results are illustrated in Fig. 1.

Discussion

It is probable that if a constant result of renal tubular damage is a considerable increase in the excretion of a substance by the damaged kidney, that substance is normally reabsorbed by the portion of the tubule affected. On the other hand a regularly occurring decrease in excretion, significantly greater than that which reasonably can be ascribed to increased tubular permeability, justifies the conclusion that the affected segment of the tubule is normally a site of active excretion of the substance in question.

Before interpreting observed changes in this way, however, one must be reasonably certain of several factors: that any changes in excretion of the reference substance (creatinine), which normally does not pass through the wall of the tubule in either direction, are due to back diffusion through the damaged cells; that the rate of back diffusion of the substance under investigation (phosphate in this instance) does not differ sufficiently from that of creatinine to account for any diminution of excretion greater than that observed for creatinine; that the anatomical damage can be localized with reasonable precision; and that the sites of anatomical and physiological damage correspond fairly closely.

The observation that during osmotic diuresis the creatinine clearance by kidneys with either tartrate nephrosis or distal nephrosis rose with increasing urine volume until it approximated the clearance by the normal kidney in the same animal, and that the phosphate excretion from kidneys with tartrate nephrosis is affected in a similar manner, points to the probability that back diffusion is the main, if not the sole, factor concerned in the reduction of creatinine clearance in both tartrate and distal nephrosis, and in the reduction in phosphate excretion in tartrate nephrosis. On the other hand, the slight effect of osmotic diuresis on phosphate excretion in distal nephrosis suggests that back diffusion plays a minor role in the reductions in phosphate excretion observed with this lesion.

The areas of anatomical damage can be localized quite readily in isolated tubules. The lesions produced by dichromate and tartrate may overlap to a certain extent as it is impossible to measure the damaged area or the length of the proximal tubule with any degree of precision. Further, as is well known, the tubules vary considerably in length. However, the observable damage produced by dichromate is confined to the first portion of the proximal tubule and involves considerably less than one half of that portion of the nephron, whereas tartrate affects the lower portion of the proximal tubule and damages more than half that segment. The lesions produced by retrograde injection up the ureter were definitely confined to the distal segment. No damage could be detected in the loop of Henle or the proximal tubule.

It is not possible to prove definitely that the physiological and biochemical lesions are located in the same areas as the anatomical damage, but when the morphological lesions in different segments of the tubule produce distinctly different functional changes it seems improbable that there can be any marked disparity between the anatomical and functional lesions. Further, the changes in functions which have been precisely located by micropuncture are associated with anatomical lesions in the areas which have been identified as the sites of those functions (Table I). Over seventy-five per cent of the glucose is reabsorbed in the first quarter to one third of the proximal tubule in the amphibian and in the mammal (27, 28), and it is only in dichromate nephrosis, which produces lesions confined to about the first third of the proximal tubule, that any significant increase in glucose excretion is observed. In tartrate nephrosis, affecting approximately the last two thirds of the proximal tubule where the remaining small amounts of glucose are reabsorbed, small increases in glucose excretion are found. No significant change in glucose excretion is found in distal nephrosis. Ammonia secretion, which has been shown in the amphibian to be confined to the distal segment (29), is sharply reduced by the lesions in the distal tubule. The excretion of sodium and chloride, which are reabsorbed along the whole course of the tubule (28, 30, 31), are increased by all three lesions studied in this investigation. The excretion of potassium, which is reabsorbed by the proximal tubule and excreted by the distal tubule (31), is increased in both dichromate and tartrate nephrosis and, under conditions of ordinary potassium intake, slightly decreased following distal damage. This correlation between the physiological and morphological changes in respect to those functions which have been definitely located anatomically increases the probability that the locations of the functional changes and the anatomical lesions observed in this investigation correspond.

Taking into consideration all the above factors, it appears reasonable to assume that the finding that damage to the first portion of the proximal tubule always results in increased renal excretion of phosphate, while distal tubular damage invariably reduces the output of phosphate by the kidney, means that phosphate is reabsorbed, partially at least, in the first part of the proximal tubule and actively excreted somewhere in the distal segments. In view of the indefinite results of damage to the last two thirds of the proximal tubule, it is doubtful if this part of the nephron has any important quantitative role in the handling of phosphate. The fact that distal tubular damage inhibits the action of parathyroid hormone in increasing the renal excretion of phosphate while neither dichromate nor tartrate nephrosis have any effect on the action of this hormone (32) is further evidence for the active excretion of phosphate by the distal tubule.

The existence of a mechanism of reabsorption in the proximal tubule with active excretion in the distal tubule could explain many of the apparently contradictory observations on phosphate excretion. The marked variations in the Tm for phosphate in the same individual could well be due to the establishment of differing levels of distal phosphate excretion with no change in the

rate of reabsorption. The finding of Foulks (17) that much more phosphate is apparently reabsorbed when the filtered load of phosphate is increased by methods which do not involve the infusion of phosphate than when the same increase is produced by phosphate infusion, and the homeostatic adjustments resulting from fasting and from phosphate feeding reported by the same author are most readily explained by stimulation of phosphate excretion by increase in body inorganic phosphate, a possibility which is not excluded by that author. The apparent decrease in phosphate reabsorption following prolonged phosphate infusion (33, 34, 35) is also probably due to increased distal tubular excretion.

It may be noted that the results reported here show considerable variation in the excretion of phosphate by different animals. This is probably the result of variation in food intake which has been shown to affect phosphate excretion (17). Some dogs eat well following the operation, whereas others take practically nothing in the period between the renal injection and the performance of the clearances. If this variation in excretion, unavoidable under our experimental conditions, is due, as is postulated here, mainly to variations in the active distal excretion of phosphate, it would explain much of the variation in percentage inhibition of phosphate excretion observed following distal tubular damage.

It is quite possible that the proximal tubule has a maximal transport capacity for phosphate and that the phosphate Tm's that have been observed have been the result of a steady state of distal tubular excretion at the time of their estimation. A few observations in this laboratory on dogs with distal tubular nephrosis, although too few to be conclusive, suggest that the proximal reabsorption of phosphate is in fact limited by a maximal transport capacity.

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THE MODE AND SITE OF THE RENAL ACTION OF PARATHYROID EXTRACT IN THE DOG[1]

T. F. NICHOLSON

Abstract

When 20 U.S.P. units per kg of parathyroid extract were injected intravenously into dogs with unilateral distal tubular nephrosis, the phosphate excretion from the normal kidney increased significantly, but there was no change in the amount of phosphate excreted by the nephrotic kidney. In dogs with unilateral nephrosis affecting the first third or the last two thirds of the proximal tubules, parathyroid extract produced a similar increase in phosphate excretion by the normal and the nephrotic kidneys. It is concluded that the site of renal action of parathyroid extract is in the distal tubule and that its mode of action is to stimulate active excretion of phosphate.

There is a sharp divergence of opinion as to the mechanism by which parathyroid extract increases the renal excretion of phosphate by the mammalian kidney. The varying theories have been reviewed recently by Bartter (1). One theory is that the parathyroid acts to depress the rate of tubular reabsorption of phosphate, while according to the others the augmented excretion may be explained by a rise in the filtered load due to either a rise in serum phosphate, an increase in the glomerular filtration rate, or both. The possibility that parathyroid extract might stimulate active tubular excretion is practically never considered. Levinsky and Davidson (2) have recently reported that parathyroid extract causes marked increases in phosphate excretion in the fowl, in which species they have demonstrated tubular excretion of phosphate, but they do not believe that their results give definite evidence for an increase in tubular excretion rather than an inhibition of tubular reabsorption of phosphate. As recent work in this laboratory has produced evidence for active distal tubular excretion of phosphate (3), the experiments reported here were designed to investigate the possibility that parathyroid extract might affect this excretory activity of the distal tubule.

Experimental

The effect of damage to three different portions of the dog's kidney was studied. The methods of selective unilateral renal damage, urine collection, clearance procedures, and analytical methods have been described previously (3,4,5). After 1 hour of equilibration, urine was collected from each ureter for a 2-hour control period. Parathyroid extract, 20 U.S.P. units per kg (Lilly), was then injected intravenously. After a 10-minute interval urine was again collected for a 2-hour period. Blood was taken at the beginning,

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the middle, and the end of each period. The excretion of phosphate was calculated as micrograms per minute. The filtered load of phosphate per kidney was calculated using the creatinine clearance by the normal kidney on the assumption that changes in clearance by the nephrotic kidney were due to back diffusion from the damaged tubule (3, 4, 5, 6). In two of the animals with distal tubular nephrosis, instead of administering 20 U.S.P. units per kg of parathyroid extract as a single injection, the dose of extract was dissolved in 240 ml of the sustaining fluid (3) and infused at the rate of 1 ml per minute over a period of 4 hours. The phosphate excretion for the hour before the injection of parathyroid hormone and for the last hour of the injection period was determined.

Results

The effect of damage to the first third (dichromate nephrosis) and the last two thirds (tartrate nephrosis) of the proximal tubule and to the distal tubule are shown in Tables I, II, III, and IV. In Table I there are also shown the results in a dog with both kidneys normal. The question of the localization of the lesions and of the relationship of the physiological and biochemical lesions to the anatomical lesions have been discussed in the previous paper (3). Neither dichromate nor tartrate nephrosis had any significant effect on the renal action of parathyroid extract, the increment in phosphate being similar in the urines from the normal and nephrotic kidneys in the same animal. When the distal tubules were damaged, however, the increase in phosphate

TABLE I

The effect of damage to the first third of the proximal tubule (dichromate nephrosis) on the renal action of parathyroid extract

	Creatinine clearance		Excretion of inorganic P, micrograms per minute						Urine volume, ml/min	
					Increase		Serum inorg. P, mg/100 ml	Filtered inorg. P, mg/min per kidney		
Period	Norm.	Neph.	Norm.	Neph.	Norm.	Neph.			Norm.	Neph.
Control	25	26	187	220			4.1	1.04	0.18	0.23
Parathyroid*	26	26	251	281	64	61	4.0	1.04	0.27	0.36
Control	26	25	135	213			4.1	1.04	0.11	0.14
Parathyroid*	27	27	300	383	165	170	3.8	1.02	0.12	0.13
Control	31	32	237	400			5.1	1.58	0.19	0.20
Parathyroid*	28	27	422	599	185	199	5.0	1.40	0.11	0.13
Control	41	39	198	396			4.8	1.92	0.56	0.61
Parathyroid*	37	36	375	548	177	152	4.7	1.71	0.59	0.63
	Normal dog									
	Right	Left	Right	Left	Right	Left		Right	Left	Right	Left
Control	21	23	169	166			4.2	0.88	0.97	0.12	0.12
Parathyroid*	25	24	284	286	115	120	3.7	0.92	0.89	0.20	0.21

*20 units per kg of parathyroid extract intravenously, 10 minutes before the start of this period.

TABLE II

The effects of damage to the last two thirds of the proximal tubule (tartrate nephrosis) on the renal action of parathyroid extract

Period	Creatinine clearance		Excretion of inorganic P, micrograms per minute					Serum inorg. P, mg/100 ml	Filtered inorg. P, mg/min per kidney	Urine volume, ml/min	
			Ratio, norm./ neph.			Increase					
	Norm.	Neph.		Norm.	Neph.	Norm.	Neph.			Norm.	Neph.
Control	40	21	.52	223	110			4.6	1.84	0.21	0.40
Parathyroid*	37	18	.49	404	260	181	150	5.0	1.85	0.25	0.51
Control	32	15	.47	148	73			5.2	1.66	0.36	0.54
Parathyroid*	30	15	.50	390	292	242	219	5.1	1.53	0.32	0.56
Control	26	16	.61	482	275			5.8	1.51	0.15	0.37
Parathyroid*	27	15	.55	654	340	172	165	5.6	1.51	0.22	0.73
Control	38	20	.53	302	149			4.8	1.82	0.53	0.78
Parathyroid*	36	21	.58	463	258	161	149	4.6	1.66	0.30	0.79

*20 units per kg of parathyroid extract intravenously, 10 minutes before the start of this period.

TABLE III

The effect of distal tubular damage on the renal action of parathyroid extract

Period	Creatinine clearance		Excretion of inorganic P, micrograms per minute					Inorg. P, mg/100 ml	Filtered inorg. P, mg/min per kidney	Urine volume, ml/min	
			Ratio, norm./ neph.			Increase					
	Norm.	Neph.		Norm.	Neph.	Norm.	Neph.			Norm.	Neph.
Control	20	6	.30	127	9			6.4	1.28	0.16	0.78
Parathyroid*	21	7	.33	208	9	81	0	6.3	1.29	0.15	0.80
Control	34	21	.68	381	137			5.8	1.97	1.11	1.97
Parathyroid*	31	19	.61	502	121	121	−16	5.2	1.61	1.15	2.03
Control	28	19	.68	236	132			6.3	1.63	0.43	0.79
Parathyroid*	21	14	.67	349	144	113	12	6.0	1.26	0.48	0.85
Control	27	18	.67	272	98			5.7	1.56	0.65	0.91
Parathyroid*	25	15	.60	498	96	226	−2	5.5	1.38	0.69	1.02
Control	34	29	.85	192	112			3.9	1.33	0.44	0.59
Parathyroid*	31	26	.84	375	155	183	43	4.0	1.32	0.47	0.61

*20 units per kg of parathyroid extract intravenously 10 minutes before the start of this period.

TABLE IV

The effect of distal tubular damage on the renal action of parathyroid extract

	Creatinine clearance		Excretion of inorganic P, micrograms per minute				Inorg. P, mg/100 ml	Filtered inorg. P, mg/min per kidney	Urine volume, ml/min	
					Increase					
	Norm.	Neph.	Norm.	Neph.	Norm.	Neph.			Norm.	Neph.
Control	30	20	195	79			5.7	1.71	0.54	1.03
Parathyroid*	31	22	576	76	381	−3	4.8	1.49	0.98	1.76
Control	29	18	254	103			5.4	1.57	1.10	1.84
Parathyroid*	31	20	699	118	445	15	4.7	1.46	1.21	2.01

*20 units per kg of parathyroid extract intravenously infused over a 4-hour period. Phosphate excretion determined for 1 hour before infusion and for the last hour of the infusion.

excretion following intravenous injection of the extract was almost completely abolished when the damage to the tubule was such that the back diffusion of creatinine was sufficient to reduce the clearance to 68% or less of the normal. Even when the creatinine clearance by the nephrotic kidney was as high as 84% of the normal, the augmentation of phosphate excretion from the damaged kidney was only 24% of that from the normal kidney. In no instance could the change in phosphate excretion following the administration of parathyroid extract be ascribed to an increase in the amount filtered.

Discussion

The most probable explanation of the finding that when the distal tubule was damaged there was a definite inhibition of the renal action of parathyroid extract and that proximal tubular damage had no such effect, is that the site of action of the extract is in the distal segment. Further, as the distal tubular lesion also results in sharp decreases in the excretion of phosphate by the kidney (3), it is most likely that the activity of the distal tubule controlled by parathyroid extract is excretion rather than absorption, and the role of the extract is stimulation of that excretion, rather than inhibition of reabsorption. This brings the activity of parathyroid extract on the kidney into conformity with that of hormones in general which, in so far as their functions have been reported, facilitate or augment the activities which they influence.

Under most circumstances the amount of phosphate excreted in the urine is less than the amount filtered. This is probably due to the reabsorption of phosphate, which as has been shown in the previous paper (3) takes place mainly in the first part of the proximal tubule, being greater than the distal excretion. Under conditions of phosphate loading we have found, in experiments to be reported later, that the excreted phosphate may some times exceed the filtered load. The observation agrees with those of a few other investigators (7,8).

Whether or not the action of parathyroid extract in stimulating phosphate excretion by the distal tubule is due to the same hormone as regulates the level of serum calcium or to a separate secretion of the gland is not established by these experiments, nor is the role of the parathyroid in phosphate homeostasis determined. The fact that, in normal individuals, raising the level of serum calcium, which presumably inhibits parathyroid activity in respect to calcium, results in a diminution of the renal excretion of phosphate (9,10) might be taken as indicating that the same hormone is responsible for the effect on serum calcium and on urinary phosphate, but proof, one way or the other, is lacking. There appears to be an active excretion of phosphate by the distal tubule which is controlled, to some extent at least, by the parathyroid. It is likely that this mechanism plays a part in regulating the level of phosphate in the extracellular fluid.

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THE ELECTROPHORETIC ANALYSIS OF PLANT VIRUS PREPARATIONS[1]

P. M. TOWNSLEY

Abstract

Zone electrophoresis in agar gel has been used for separating tobacco mosaic virus and potato virus X from crude plant extracts and reaction mixtures. The viruses were located within the gel by autoradiographic, serological, and protein-staining methods.

Introduction

The study of the intermediary metabolism of plant viruses demands the use of a technique which would separate the intermediates from reaction mixtures rapidly yet gently. The separation of serum proteins by electrophoresis in agar gel (1) suggested that this matrix might be used for virus studies.

The following investigation presents the successful use of this method for the resolution of tobacco mosaic virus (TMV) and potato virus X (PVX) infection, the separation of radioactive metabolic intermediates, and the location of serologically active compounds.

Materials and Methods

The Reco electrophoresis chamber,* model E-800-2, was modified for continuous buffer renewal by replacing the carbon electrodes with platinum wire contained within a $\frac{1}{4}$-in. dialysis tube. The electrode reaction products were removed by a slow passage of buffer through the dialysis tubing.

Stock $2\frac{1}{2}\%$ Difco Bacto agar was prepared by dissolving it in distilled water at 100° C, followed by cooling in a shallow tray, dicing, and finally dialysis against distilled water. The agar electrophoresis bed, $34 \times 21 \times 0.6$ cm, was prepared by melting the stock agar with distilled water and tris(hydroxymethyl)aminomethane maleate (tris maleate), pH 7.0, buffer to give 0.7% agar with a final buffer molarity of 0.02. The melted agar was then cooled to 50° C and poured on to the water-cooled electrophoresis bed. Before the agar was poured a celluloid sheet was placed on top of the bed to facilitate the removal of the agar after the electrophoretic analyses. After the agar had solidified, narrow slots, 3 mm wide, having approximately twice the capacity of the volume of the sample were cut into the agar transverse to the electrical field and 6 cm from the cathode end of the gel. A hot spatula was inserted into the empty slot and sufficient agar melted to seal the bottom to prevent the sample from running under the agar.

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Contribution No. 5, Chemistry Division, Science Service, Canada Department of Agriculture, Vancouver, B.C.

*Research Equipment Corporation, Oakland, California, U.S.A.

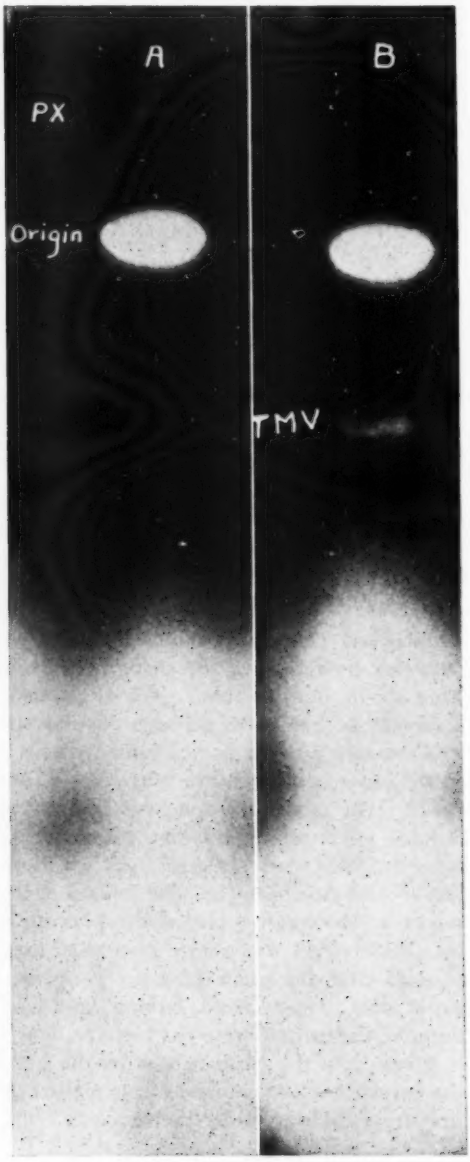

FIG. 1. Autoradiogram of PVX and TMV in extracts from *Nicotiana tabacum* L. var. Haronova. Agar electrophoresis (4.4 volts per cm), for 24 hours in 0.02 *M* tris maleate buffer, pH 7.0, of 20 μl of crude young leaf extract after 8 hours' incubation of the leaves in P^{32}.

For large-scale preparative purposes a strip of Whatman No. 3 mm paper was placed under the origin location of the sample before the agar was poured. This paper acted as a seal for the sample permitting the omission of the heat-sealing step, a difficult procedure for large samples.

Tobacco plants, *Nicotiana tabacum* L. var. Haronova, were inoculated with either TMV or PVX. Two weeks later the young top leaves were removed for analysis. They displayed the characteristic appearance of the virus infection.

For P^{32}-incorporation studies, leaves were immersed by their petioles in Hoagland's solution supplemented with Haas and Reed's trace element solution.* Ten microcuries of P^{32} orthophosphate per milliliter were added to the nutrient solution and the leaves were permitted to photosynthesize under the mixed artificial and daylight illumination in the greenhouse. A sample of the leaf juice was expressed at 0° C with a mortar and pestle and an aliquot of this juice was pipetted immediately into the slots for electrophoretic separation. The electric field strength was 4.4 volts per cm (150 volts difference of potential at the electrodes with a current of 14 ma).

Upon completion of the electrophoretic separation the virus was located either by a variation of the serological gel diffusion precipitin test (2), by drying agar (3) into a thin sheet suitable for autoradiography (4) or for staining with Solway purple (5).

Serological precipitation was performed either on an extract from the gel (6) or on the gel itself. In the latter case a narrow strip of filter paper soaked in the appropriate rabbit antiserum was laid on the top of the gel. Incubation was necessary to permit diffusion of the antibody into the gel; usually 12 hours elapsed before a precipitin reaction was visible.

Autoradiographic analyses were made using Kodak Medical "No-screen" X-ray film.

Results

I. The Location of TMV and PVX in Crude Extracts by Autoradiographic, Serological, and Protein-Staining Methods

Extracts from healthy and TMV-diseased P^{32}-labelled leaves were separated electrophoretically, in 0.02 *M* tris maleate pH 7.0 buffer, into four groups toward the anode in the following order: the origin, a broad band extending from the origin containing nucleoprotein and proteins, nucleic acids, and inorganic phosphate. Known samples of protein, nucleoprotein, nucleic acid, and phosphate were used to confirm the positions of these general groups. Tobacco mosaic virus was located by staining with Solway purple or by autoradiography as a fast-migrating material in the nucleoprotein fraction (Fig. 1A). There were, no doubt, many other unidentified materials dispersed throughout the agar gel. The location of TMV antigen by serology indicated that the general area corresponding to TMV in the autoradiogram contained at least two immunologically active components, designated in

**Cited in* Curtis, O. F. and Clark, D. G. An introduction to plant physiology. McGraw-Hill Book Co. Inc., New York. 1950. p. 384.

this paper as the TMV band. No serological reaction was observed with healthy juice. Both the TMV band and the origin carried infective particles as shown by their ability to infect *Nicotiana tabacum* L. var. Haronova. No other area was tested, but conceivably the nucleic acid band may also be infective. The infectivity at the origin could be due to virus contained within and adsorbed to cell debris.

At pH 7.0, PVX migrated toward the cathode and was readily registered as a narrow radioactive band (Fig. 1B). Within a broad serological precipitin area, a zone, strongly stained with Solway purple, overlapped the radioactive band. The movement of PVX toward the negative pole at pH 7.0 provided an excellent method for the isolation and study of this virus. The localization of the incorporated P^{32} as a narrow band, which did not diffuse during the drying of the gel, suggested that the isotope was incorporated into large molecules.

The ease and the completeness of the separation of TMV and PVX from crude juices indicated that an electrophoretic technique could be used as an aid in metabolic studies of these two viruses.

The radioactivity associated with a component of the TMV band did not appear to be a mere sorption of the labelled nucleic acid constituents from healthy plant juice with TMV protein. This was tested by mixing equal portions of P^{32}-labelled tobacco juice from healthy plants with non-labelled TMV infected tobacco juice, by incubating the mixed preparation for 5 minutes at room temperature, and then by subjecting the mixture to electrophoretic separation. No radioactivity over background was noted in the area corresponding to TMV. Furthermore, no radioactivity was located in either the TMV band or in the PVX band when the respective diseased leaves were macerated with P^{32}-labelled healthy leaves and submitted to electrophoresis.

In 0.02 *M* tris hydrochloric acid buffer, pH 8.0, PVX located autoradiographically moved approximately one-half the distance travelled at pH 7.0. On the other hand, the migration of TMV at pH 8.0 was not noticeably altered.

II. The Incorporation of Radioactive Orthophosphate into Leaves Excised from TMV-Diseased and Healthy Tobacco Plants with Increasing Time of Incubation

It was desirable to know the approximate time at which TMV became labelled with P^{32} under the conditions of the experiment, as well as to determine the possibility of locating transient intermediates which may have been masked after long incubation with radioactive phosphorus.

Radioactivity could be detected within a component of the TMV band after the leaves had been allowed 2 hours' uptake in P^{32} solution. Increasing the time to 8 hours indicated that no other area within the TMV band incorporated the radioactive phosphorus at significant levels above the background established for healthy leaves (Fig. 2). As with PVX the incorporation of P^{32} into a narrow radioactive area indicated a slow diffusion rate and

thus the probability that the isotope was incorporated into large particles.

After short periods of uptake and before the P^{32} could be detected in the TMV band there was a marked increase in radioactivity both at the origin and at the nucleic acid band which exceeded that of the juice of healthy, control plants. Whether the increase was due to intermediary or to degradation compounds related to TMV remains to be determined. This radioactivity, however, need not be the result of viral intermediates, since the uptake of P^{32} and its metabolism under the conditions of the experiment was greatly accelerated in the diseased leaves.

III. The Stability of the P^{32} TMV Band to Citrate Ions

Tobacco mosaic virus prepared by mild ultracentrifugation contains, in addition to the virus, large amounts of material possessing the properties of normal leaf nucleoprotein (7, 8). This nucleoprotein can be modified by more vigorous methods of virus purification such as freezing, salt precipitation, and incubation with phosphate or citrate ions so that it no longer resembles TMV.

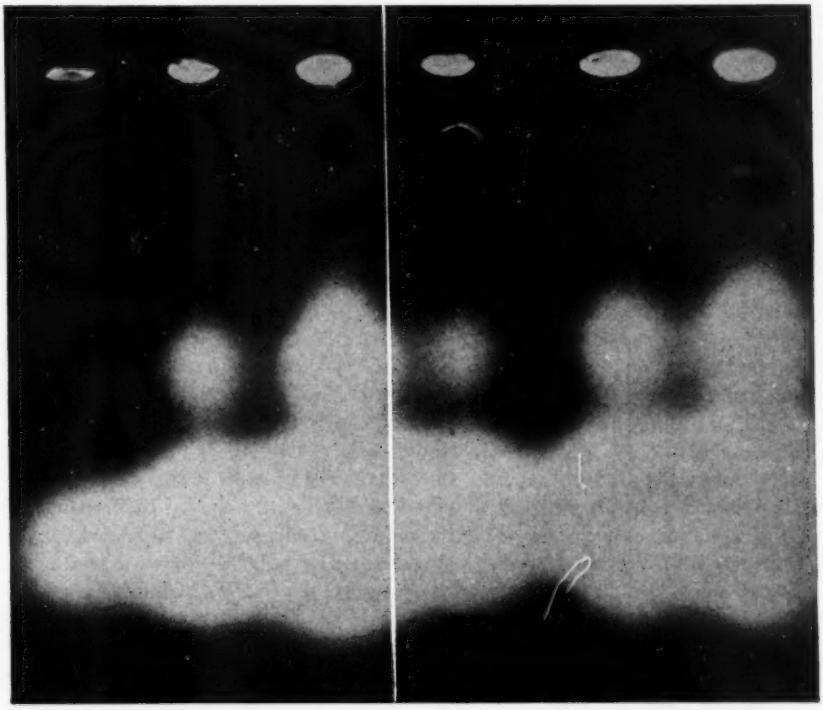

FIG. 2. Autoradiogram showing the accumulation of P^{32} in healthy and TMV-diseased *Nicotiana tabacum* L. var. Haronova leaf extracts. Agar electrophoresis, as for Fig. 1, of 20 μl of crude leaf extracts after 4, 6, and 8 hours' incubation of the leaves in P^{32}. Left: healthy; right: diseased.

Preliminary experiments on the stability of the incorporated P^{32} in the ultracentrifuged preparations of TMV to 0.012 *M* citrate ions also indicated that a high proportion of the P^{32} was labile and was degraded to material which was soluble in 5% trichloroacetic acid. This raised the question as to whether or not the P^{32} incorporated into the TMV band after a short period of uptake by the excised leaves was actually citrate-sensitive, normal nucleoprotein.

A preparation of TMV obtained by alternate slow- and high-speed centrifugation (7) of juice from leaves that had been allowed P^{32} uptake for 4 hours was analyzed electrophoretically after the following treatments. Aqueous solutions of the virus pellet were incubated for 12 hours in 0.012 *M* citrate at 0° C, and at 37° C. Another aliquot of the pellet solution was incubated at 0° C in the citrate solution containing 3×10^{-3} *M* zinc acetate to inhibit leaf ribonucleose activity (9). In addition, aliquots were analyzed at once without further treatment, and after 12 hours' incubation at 0° C. A drop of chloroform was added to each preparation to inhibit bacterial growth (7). The results of the electrophoretic analysis are shown in Fig. 3. The electrophoresis of the untreated aliquots indicated the presence of three radioactive zones within the TMV band which were not present in healthy nucleoprotein pellets. Because of the presence of one zone only in fresh juice it was thought that these zones may represent virus aggregation or possible degradation as the result of ultracentrifugation. Incubation of the TMV preparation

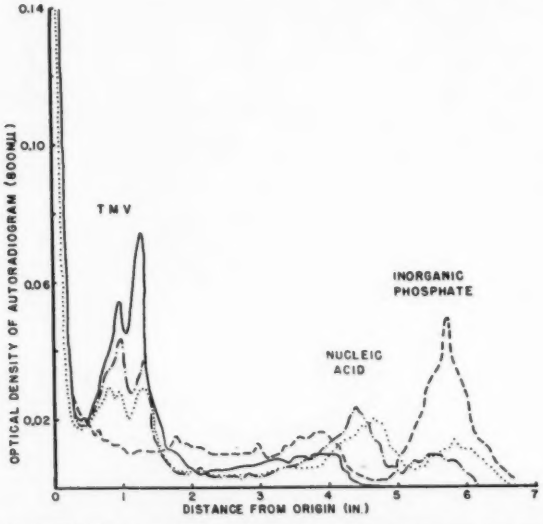

FIG. 3. The instability of TMV labelled with P^{32} to ultracentrifugation and citrate treatment. The TMV pellet was taken up in 3 ml H_2O and 0.5-ml aliquots removed for treatment. ——— + 0.1 ml H_2O, incubation for 12 hours at 0° C; . . . . . + 0.1 ml 0.25 *M* sodium citrate, pH 7.0, incubation for 12 hours at 0° C; —·—·— + 0.1 ml 0.25 *M* sodium citrate, pH 7.0, + 3×10^{-3} *M* zinc acetate, incubation for 12 hours at 0° C; – – – – – + 0.1 ml 0.25 *M* sodium citrate, pH 7.0, incubation for 12 hours at 37° C. After incubation 20-μl aliquots were removed for analysis as in Fig. 1.

at 0° C with citrate ions resulted in a decrease in the radioactivity found in the TMV bands and the appearance of radioactivity in the nucleic acid and inorganic phosphate bands. The presence of zinc ions did not alter the activity found in the nucleic acid and phosphate zones but did tend to prevent some decrease in the radioactivity in the TMV bands. The slightly greater mobility of the nucleic acid and inorganic phosphate in the preparation which was not treated with zinc was thought to be due to a local change of electric field in the agar gel. The reason for the apparent protection of TMV by zinc was not known.

Incubation of the TMV preparation at 37° C with citrate ions resulted in the almost complete loss of radioactivity from the TMV bands and the appearance of radioactivity at the site corresponding to inorganic phosphate. No P^{32}-labelled nucleic acid band was released by this process. The optical density of the autoradiogram at the sample origin could not be estimated. However, it would appear quite possible that the radioactivity lost from the TMV band might have been located at the origin and that the nucleic acid and inorganic phosphate bands were derived from normal or non-viral contaminating nucleoprotein.

Estimation of protein in the TMV band by a visual comparison with the control preparation of the intensity of the stain in the dried agar gel showed that the observed decrease in radioactivity was accompanied by a marked decrease in protein with a corresponding increase of protein at the origin. No protein corresponding to the TMV band could be observed in the sample treated at 37° C, but there was a considerable increase of protein at the origin in this experiment.

The results of the experiments on juice centrifuged alternately at high and low speeds appear to be complicated by the occurrence of several changes in virus mobility which may be due to simple aggregation, breakdown, or denaturation. The altered virus may or may not be susceptible to ribonuclease known to be present in ultracentrifuged preparations (5).

In order to obtain a sample of P^{32}-labelled TMV with a mobility in agar gel similar to that observed for TMV from fresh plant juice, TMV was isolated by electrophoresis in agar gel after exposure of the diseased leaves to $P^{32}O_4^{\equiv}$ uptake for 4 hours as previously described. Removal of the TMV from the agar gel was accomplished by permitting the virus to migrate through the gel to a buffer-filled trough which had previously been cut in the agar between the nucleic acid band and the virus. The location for the trough could be readily determined by radioactivity measurements. Aliquots of the TMV solution, taken after concentration by evaporation through a dialysis bag, were treated as follows: no citrate and incubation at 0° C; 0.1 *M* citrate and incubation at 0, 25, 30, and 35° C, respectively. After an incubation period of 1 hour the samples were immediately analyzed electrophoretically.

The sample, which was not incubated with citrate ions, migrated on electrophoresis at pH 7.0 in 0.02 *M* tris maleate buffer as two radioactive bands, a major band followed closely by a very faint band. The appearance of two bands rather than the one normally found from fresh crude juices from

diseased plants suggested that some change had taken place in the virus during preparation, possibly during concentration. The presence of citrate ion, regardless of the temperature, greatly increased the radioactivity in the slow-moving band. As the temperature of incubation was increased to 35° C the radioactivity of the first two bands decreased with the appearance of a third slower-moving, broad radioactive band. The amount of protein, as determined by visual comparison of the stained gel preparations, appeared to parallel the location and intensity of the radioactivity. No definite occurrence of the inorganic phosphate and nucleic acid bands was observed, indicating that the bands found in the previous experiment probably arose from unstable contaminating nucleoprotein.

Conclusion

The separation of soluble plant extracts into a number of components by electrophoresis in agar gel enables the method to be used not only to fractionate and purify tobacco mosaic virus (TMV) and potato virus X (PVX), but also to follow the chemical changes that occur in biological systems.

Radioactive phosphate appears to be rapidly incorporated into a particle of large molecular weight which is probably TMV. The TMV band on the agar gel was found to be labelled within 2 hours' uptake by young leaves, which suggested that the virus was synthesized at a rapid rate considering the dilution from unlabelled precursors. Such a conclusion is in agreement with that reached by Stephenson *et al.* (10) from studies of leucine-C^{14} incorporation by the protein moiety of TMV. These authors found that the virus became labelled after 2 hours' uptake under experimental conditions comparable to those of this investigation.

The occurrence in ultracentrifuged TMV preparations of P^{32} activity which was unstable on exposure to citrate ions was thought to be derived from contaminating normal nucleoprotein. It did not appear to be derived from an unstable particle combined with or electrophoretically related to TMV.

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EFFECTS OF CONTRACTILE STIMULI ON EXCHANGES OF ELECTROLYTES BETWEEN UTERINE TISSUES AND A SALINE–BICARBONATE MEDIUM[1]

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With the technical assistance of
JEAN MCCARTNEY and K. ROBINSON

Abstract

(1) Evidence has been presented suggesting that progesterone pretreatment in vivo increases the rate of leakage of potassium from rabbit uterine segments into a saline–bicarbonate medium.

(2) Various types of evidence indicate that in uterine tissues, the loss of potassium from cells into potassium-free solutions is increased during the contractile actions of drugs. There was no clear-cut concomitant increase in tissue sodium concentrations. The results tend to confirm previous findings indicating that increases in potassium (but not in sodium) permeability are associated with contraction of smooth muscle.

(3) Epinephrine effects on potassium loss occurred only in uteri which contracted in response to epinephrine. Norepinephrine, but not iproterenol, had a similar effect. Dibenamine, alone, decreased potassium loss and partly blocked the effects of epinephrine. Direct measurement of glycogen content suggested that these findings were not related to a glycogenolytic action of the epinephrine. Rather, the effect of epinephrine to increase potassium loss seemed to be closely related to its contractile action. In long-term experiments, both epinephrine and calcium slowed the rate of K loss and of water and sodium gain. This effect also was absent in uterine tissues which were not contracted by epinephrine.

Introduction

Rabbit and cat uteri previously have been found (1, 2) to contain a cellular concentration of potassium as high as or higher than that in other contractile tissues. Uterine chloride is present chiefly in the extracellular space. Additional tissue chloride can be accounted for either on the basis of chemical combination or on the basis of penetration into cells according to the electrochemical gradient. In addition to its extracellular content, tissue sodium also is present partly in a site from which it can diffuse slowly (presumably cellular) and partly in a site from which it cannot diffuse even after prolonged exposure to various sodium-free solutions.

Under appropriate circumstances, spontaneous contractions of uterine smooth muscle in vitro are accompanied by action potentials which are not altered in magnitude by reduction of the sodium concentration of the medium to as little as 15–20 meq/liter (3, 4). The nature of the ionic currents and the changes in membrane permeability which are responsible for these action potentials have not been clarified. In the present study, cellular potassium depletion of uterine strips was induced in vitro by immersing the tissues in a medium initially free of both potassium and calcium (saline–bicarbonate).

[1]Manuscript received July 8, 1958.
Contribution from the Department of Pharmacology, University of British Columbia, Vancouver, B.C.

Utilization of a potassium-free medium was necessary in order to get measurable changes in external potassium concentration and to study changes in potassium outflux relatively uncomplicated by simultaneous changes in influx. Other ions (e.g. Ca and PO_4) were also omitted from the basal medium in order to permit their effects on potassium exchange to be studied independently from the effects of drugs. In this medium, impaired contractility and paralysis ultimately develop. However, contractile responses to drugs can be induced during the initial 30–60 minutes of exposure. The present report is concerned with the exchanges of cellular sodium and potassium during drug-induced contraction in saline–bicarbonate medium.

Methods

Animals and Pretreatment

Immature rabbits were treated either with estrogen alone (diethylstilbestrol in oil, 100 μg/day) subcutaneously for 6 days or for 3 additional days with progesterone in oil (2 mg/day) as well. Similarly treated or pregnant cats also have been used for comparative purposes.

Tissue Preparation

Tissues were removed from the animal as previously described (1) and rapidly cut up into pieces of approximately equal size. Uterine segments were not slit open except in the case of uteri from pregnant cats which were opened and cut into strips. Segments were weighed initially in most cases and then placed in Krebs–Ringer solution at 37° C. The tissues were oxygenated with 95% O_2–5% CO_2 for 20 minutes to permit recovery from the anoxia attendant upon isolation procedures. Tissues at the termination of this period as compared with freshly isolated uteri manifested very little alteration in water and electrolyte concentration, other than a slight increase in extracellular fluid. The tissues were then transferred to the oxygenated potassium-deficient medium. Subsequent exchanges of electrolytes between medium and tissue were examined by analyses of the medium and in some cases, the tissues as well. The potassium-deficient medium contained (in mM/l.) Na, 156.5; Cl, 136; HCO_3, 20.5; and glucose, 49.

The tissues in potassium-deficient media were handled in one of three ways:

(*1*) *Sampling procedure — delayed drug addition.*—Each piece was to serve as its own control. Several intervals were studied before addition of drugs to permit equilibration of extracellular electrolytes and to allow control values to be obtained. After each interval (duration 7–15 minutes) the piece was transferred to a fresh prewarmed solution. Before marked potassium depletion had occurred (about 45 minutes) the piece was transferred to a solution containing a stimulant drug. Several further intervals in drug-free media also were studied before the tissue was weighed and placed in the oven. Other segments of the same uterus were also run without drug treatment and served as additional controls.

(*2*) *Sampling procedure — immediate drug addition.*—When other pieces from the same uterus were to serve as controls the piece was transferred

directly from Krebs–Ringer solution into the saline–bicarbonate medium to which the drug had been added. After an appropriate interval, the solution was exchanged for fresh drug-free solution or in some cases, the tissue was weighed immediately thereafter. Usually the tissue was exposed to the drug for 10 minutes and then to drug-free saline–bicarbonate solution for 60 minutes. In some cases the contractile effects of the drugs used were verified by noting the length of pieces measured before and after exposure to the drugs.

In both procedures, analyses were made of media and tissues. Both of these procedures (1 and 2) had disadvantages. In the former, the duration of the period required for equilibration of extracellular electrolytes and getting control values was sufficient to raise doubts as to whether tissues were still capable of contraction. In the latter, the effects of drugs on exchanges across cellular membranes were diluted and may have been swamped or distorted by the extensive early loss of potassium from extracellular spaces which occurred during the period of drug actions. Both methods suffered from a more important disadvantage. Contractions may not have persisted throughout the 10-minute or more sampling intervals and effects on potassium exchange during contraction may have been obscured by opposite changes during relaxation. Attempts to shorten the sampling intervals to less than 7–10 minutes resulted in increased errors and greater variation in values for control intervals. For the above reasons, a third method was devised.

(*3*) *Perfusion technique.*—A segment of uterus was suspended between muscle clamps attached for recording isometric tension (RCA transducer 5374) in a tube surrounded by a water jacket through which water was circulated at 37° C. Prewarmed solution passed through the reservoir at a constant rate for each experiment (from 2 to 5 ml/minute in various experiments). After recovery from procedures of isolation as demonstrated by adequate contractile function, the system was flushed out with a K-free Krebs solution. In this series of experiments the potassium-deficient medium was identical with Krebs–Ringer except for the omission of potassium. Samples of the effluent were taken for 1-minute intervals. After the first 5 minutes they were found to contain relatively constant quantities of potassium. Therefore 6–10 minutes after switching to K-free Krebs solution, a drug dissolved in the same medium was added to the perfusion fluid at the point where this fluid entered the tube surrounding the tissue. A 30-second sample was collected during the rising phase of the contraction and 1-minute samples at intervals thereafter as long as the contraction persisted (up to 10 minutes) and during relaxation. This method proved the best of those used for differentiating drug effects, but did not permit comparison of tissue electrolytes to those of control pieces, treated similarly except for the addition of the drug.

Analysis of Media and Tissue

Samples of medium were diluted to appropriate volume and analyzed by methods previously described (1). When the first two methods were used the results were then multiplied by a figure relating the sample volume to the

total volume. This procedure, rather than an attempt to recover and analyze all of the medium was adopted because of the almost inevitable loss of medium adhering to tissue and suspension tube. It was shown that exchange of water from or into the tissue, the chief source of error in this procedure, did not alter the volume of the potassium-deficient medium appreciably. In all cases studied volume changes amounted to less than 0.01 ml per ml of initial sample. Carry-over of solution adhering to the tissue probably occurred, but this was kept nearly constant by rigid adherence to details of the mechanics of transfer.

Dried tissues were analyzed by methods described previously (1). Glycogen was isolated by the method of Good *et al.* (7) and measured by the anthrone method of Young and Raiz (8).

Controls and Intra-uterine Variation

In experiments in which effects on tissue electrolyte concentration were studied (sampling procedures 1 and 2), the experimental values were compared to the relevant controls in that experiment. All values obtained have been expressed as the difference (experimental value minus control value).

Electrolyte analyses of uterine pieces cut from various areas revealed significant differences of the following types. (1) Variations related to position in the horn; for example, pieces near the vagina often contained more potassium and less sodium per unit wet weight than the piece next to the Fallopian tube. (2) Variation between horns; for example, one horn often had more mass than the other, or its pieces sometimes contained more potassium per unit mass than corresponding pieces from the opposite horn. Various experimental designs were adopted to permit such variation to be detected and minimized. In general, tissue segments were compared either with adjacent pieces in the same horn, or with symmetrically placed pieces in the opposite horn, depending upon which comparison provided a more valid comparison, as judged by control analyses of pieces taken from various portions of the uterus.

Maintenance of a constant tissue mass – fluid volume ratio was essential in the sampling experiments. The smaller this ratio, the greater the potassium loss tended to be (see Fig. 1).[2] Therefore, a volume of 5 ml was used in almost all experiments, and pieces from a given uterus (to be compared in a single experiment) were cut as nearly as possible so that all had an equal mass. Experimental pieces whose mass differed by more than 20% from the control selected to minimize intra-uterine variation (see previous paragraph) were excluded. If the mass of the experimental piece differed by more than 10% from the preferred control, the values were included only when another control piece of similar weight was also available for comparison and showed similar differences.

Effects of drugs on cat uterine tissues could not be studied efficiently by sampling methods despite attempts to rule out various extraneous sources of

[2]When the volume of the fluid was varied concomitantly with the mass to maintain a constant ratio, the potassium remained relatively constant. This was probably the result of the accumulation of potassium in the external medium leading to an appreciable influx of this ion. Presumably the influx of potassium was directly related to the external concentration. A similar conclusion was drawn from experimentation in which the duration of the sampling interval was varied. The shorter the sampling interval, the faster was the rate of net potassium loss.

variation. Wide, inherent variation between pieces from the same uterus in this species necessitated use of methods in which each piece served as its own control. Only a few selected experiments of this type on cat uterus have been included when apparently valid comparisons could be made.

Electrical Stimulation

Electrical stimulation was carried out in some experiments. The procedure of Csapo and Corner (11) was used. The strip was stimulated every 30 seconds for the period of drug exposure through platinum electrodes tied at each end of the uterine strip. Sixty-cycle a-c. current was applied for 5 seconds at a voltage varying from 20 to 30 volts dependent upon the length of the uterine strip.

Results

Effects of Hormonal Pretreatment on Cation Exchange during Exposure to Saline–Bicarbonate (without Added Drugs)

Table I includes the tissue electrolyte concentrations obtained in 30 consecutive instances in which rabbit uteri were analyzed (by the same individual) before and after exposure to the K-free medium for 70 minutes as in sampling procedure 2. (These do not include all of the values summarized in Fig. 1.) On the right side of this table, the values for intracellular concentrations have been calculated assuming that chloride space is equivalent to the extracellular space.[3]

TABLE I

Exchanges of unstimulated uterine segments in K-free bicarbonate–saline
(After previous equilibration in Krebs solution (20 minutes))

	Na_t*	K_t	Cl_t	H_2O_t	Cl_{sp}	Na_c†	K_c	$Na+K_c$
E (30)								
Control	75.4	65.6	51.6	834	473	12.2	169.4	181.6
K-free	105.5	51.9	75.9	870	557	59.8	163.2	222.0
Difference	+30.1	−13.7	+24.3	+35.7	+84	+47.6	−6.2	+41.4
S.E.	2.2	1.1	2.1	6.2				
E+P (30)								
Control	81.4	68.5	49.0	834	449	36.4	171.9	208.3
K-free	104.7	51.1	70.5	862	518	69.2	147.2	216.4
Difference	+23.3	−17.4	+21.5	+27.6	+69	+32.8	−24.7	+8.1
S.E.	3.8	1.1	2.4	5.6				

*t = Ionic concentration in meq per kilogram fresh tissue or ml per kilogram fresh tissues in the case of H_2O and Cl space.
†c = Concentration for meq/liter cellular water. Cell water estimated from chloride space.
NOTE: E = estrogen pretreated; E+P = estrogen and progesterone pretreated.

The data clearly indicated that sodium penetrated tissue water outside the chloride space in both estrogen-treated and estrogen and progesterone pretreated pieces. The potassium loss in saline–bicarbonate was greater in progesterone-pretreated pieces. It was equivalent to sodium gain only in these pieces.

[3]The chloride space undoubtedly overestimates the extracellular space, but represents a convenient approximation and permits relative changes to be distinguished. See references 1 and 2 for further discussion.

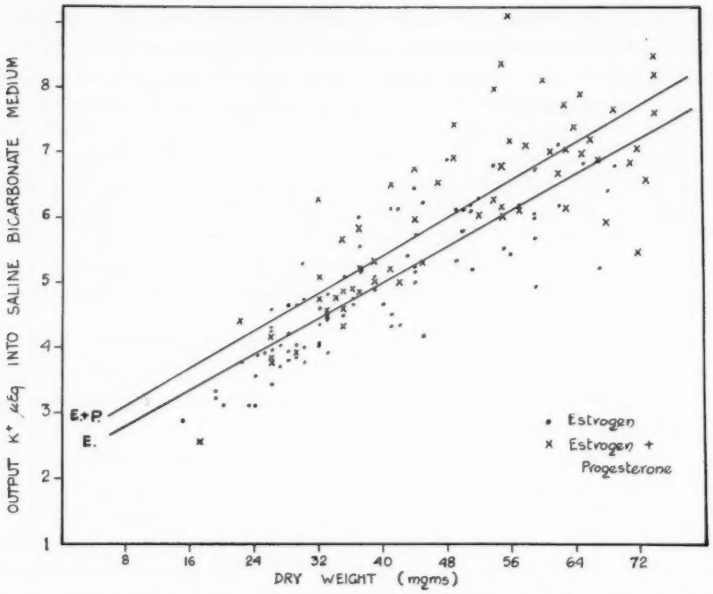

FIG. 1. Potassium loss from pieces of rabbit uterus into saline–bicarbonate medium.

This suggested that progesterone pretreatment caused an increased rate of potassium loss into K-free solutions, as compared with tissues receiving estrogen pretreatment alone. To analyze this effect further, the K outputs in all similar experiments conducted were plotted against dry weight in Fig. 1. A straight line was fitted to the data by the method of least squares and the regression of K output on weight for the two pretreatments was analyzed by the method of covariance (12).[4] This analysis indicated that even when variation due to the greater average mass of progesterone-treated pieces was excluded, the tendency for increased K loss after progesterone treatment persisted.

The data on K loss from pieces of cat uteri similarly treated were not extensive enough to permit such comparisons, but gave no indications of a similar difference. In fact, pieces from progesterone-treated animals tended to lose less potassium than pieces from animals treated with estrogen alone.

Effect of Epinephrine on Electrolyte Exchanges in Saline–Bicarbonate; Short-Term Effects

Potassium

All methods used indicated that epinephrine increased the rate of potassium loss from uterine pieces which are contracted by this drug. This effect was

[4]The F ratio (for testing the significance of adjusted means) was 15.86. This leads to a P value of $<.01$. The coefficients of regression b_e and b_{ep} were 0.0681 and 0.0715; the mean weights, x_e and x_{ep}, were 39.8 and 49.4 mg. The mean potassium outputs, y_e and y_{ep}, were 4.97 and 6.12 meq.

not observed in pieces which were inherently unresponsive to the drug or after adrenergic blockade. Norepinephrine had comparable effects but iproterenol had much smaller effects on potassium loss.

In Table II (A and B) the effects of epinephrine, norepinephrine, and iproterenol on potassium loss from rabbit uterine segments in the presence and absence of adrenergic blockade have been summarized when this was studied by procedure 2 (sampling procedure — immediate drug addition). The drugs were present only during the initial 10-minute period, and a 60-minute period

TABLE II

Drugs' effects on electrolyte exchange expressed as differences from control (experimental − control)

			K in solution, meq/kg wet weight			Tissue (meq/kg dry)			H_2O, g/kg wet wt.
			10 min†	60 min	Total	Na	K	Cl	
A. Estrogen-pretreated									
Epinephrine, 1–10 μg/ml	(14)		+2.6	−1.2	+1.3	−86	−17	—	−10
		S.E.	(0.2)	(0.4)	(0.5)	(6)	(5)		(1)
Norepinephrine, 1–10 μg/ml	(10)		+2.0	−0.5	+1.2	−68	−32	—	−8
		S.E.	(0.2)	(0.3)	(0.6)	(15)	(8)		(1.6)
Iproterenol, 10 μg/ml	(5)		+0.7	+1.0	+1.7	—	—	—	—
		S.E.	(0.2)	(0.5)	(0.5)				
Acetylcholine, 10–100 μg/ml	(7)		0.0	−1.1	−1.2	−36	+12	−24	+9
		S.E.	(0.2)	(0.3)	(0.4)	(46)	(7)	(35)	(9)
Histamine, 10–100 μg/ml	(8)		−0.3	−0.4	−0.7	+10	+11	−17	0
		S.E.	(0.4)	(0.2)	(0.5)	(21)	(7)	(19)	(16)
Pitocin 0.1 U	(5)		+0.6	−0.5	+0.1	−30	+4	+28	−6
		S.E.	(0.7)	(0.3)	(0.7)	(15)	(10)	(23)	(11)
5 U	(3)		+2.9	−0.5	−3.4	—	—	—	—
		S.E.	(1.7)	(0.8)	(4.6)				
Dibenamine, 10–100 μg/ml	(11)		−1.3	−1.5	−2.4	−25	−9	—	−4
		S.E.	(0.3)	(3.0)	(1.6)	(18)	(7)		(1)
Dibenamine + epinephrine	(6)		+0.3	+0.5	+0.9	−80	−15	—	−10
		S.E.	(0.4)	(0.6)	(0.6)	(18)	(23)		(3)
Dibenamine + norepinephrine	(6)		−0.5	−0.8	−1.3	−45	−15	—	−6
		S.E.	(0.8)	(0.4)	(0.6)	(19)	(9)		(1.5)
Dibenamine + iproterenol	(2)		+0.2	−0.4	−0.2	—	—	—	—
Electrical	(4)		+9.8	+4.6	+13.6	+38*	−116	—	−17
		S.E.	(2.2)	(1.1)	(3.2)	(30)	(54)		(5)
Epinephrine, 10 μg/ml	(4)		+2.7	−1.9	+0.8	−119	−40	—	−4
		S.E.	(0.2)	(0.6)	(0.6)	(53)	(4)	—	(13)
B. Estrogen and progesterone pretreated									
Epinephrine	(10)		+1.2	−0.6	+0.6	−82	−17.5	—	−8
		S.E.	(0.3)	(0.3)	(0.7)	(29)	(7)		(2)
Norepinephrine	(7)		+1.5	−1.1	+0.4	−46	−19.5	—	−10
		S.E.	(0.3)	(0.8)	(1.1)	(14)	(3)		(4)
Iproterenol	(3)		+0.6	−0.4	+0.2	—	—	—	—
		S.E.	(0.3)	(0.2)	(1.2)				
Acetylcholine	(9)		−0.6	−1.8	−2.4	−41	−2	−10	+8
		S.E.	(0.3)	(0.2)	(0.2)	(15)	(11)	(24)	(7)
Histamine	(8)		−0.7	−0.5	−1.3	−42	+15	+10	−13
		S.E.	(0.3)	(0.1)	(0.3)	(12)	(5)	(8)	(11)
Pitocin 0.1 U	(4)		+0.1	−0.0	−0.1	−11	+2	+18	+3
			(0.4)	(0.2)	(0.5)	(21)	(5)	(18)	(18)
5 U	(4)		−0.6	−1.0	−1.6				
			(0.2)	(0.6)	(0.4)				
Dibenamine	(9)		−0.8	−1.1	−1.7	−5.6	−11	—	−6
			(0.2)	(0.8)	(0.9)	(15)	(28)		(2)
Dibenamine and epinephrine	(6)		−0.3	−2.1	−2.3	−40	+7	—	−7
			(0.3)	(1.1)	(1.1)	(36)	(22)		(3)
Dibenamine and norepinephrine	(4)		−0.3	−0.8	−1.1	−51	−5	—	−11
			(0.2)	(0.8)	(1.4)	(28)	(6)		(5)
Dibenamine and iproterenol	(3)		+0.2	−0.2	0.0	—	—	—	—
Electrical	(4)		+5.4	+2.1	+7.5	+33	−27	—	−6
			(1.0)	(1.1)	(1.2)	(46)	(36)		(3)
Epinephrine	(4)		+2.0	−1.0	+1.1	−72	−47	—	+4
			(1.2)	(0.9)	(1.4)	(26)	(48)		(2)

*Significant loss in terms of wet weight.
†Treated during first 10 minutes only.

in drug-free medium followed. As indicated by the data on Table I and by similar data from analyses of the potassium content of the media, estrogen-treated pieces lost an average of 14 meq/kg wet weight of potassium and estrogen and progesterone treated pieces lost an average of 17.5 meq/kg wet weight in such experiments when no drugs were added. The rate of loss of potassium was greater during the initial 10-minute interval since extracellular potassium emerged during this period. This loss (3.5–5 meq/kg and 4–5.5 meq/kg for pieces with estrogen alone and with estrogen and progesterone) represented one fourth to one third of the total. Both epinephrine and norepinephrine (1–10 μg/ml) caused a 5–20% shortening of rabbit uterine segments and a marked increase (>50%) in the quantities of potassium lost from estrogen-treated pieces, during the initial 10-minute interval.

In pieces treated with progesterone, the effects of adrenergic amines were qualitatively similar though quantitatively smaller (*ca.* 25%). This difference may have been due to the smaller increases possible in tissue with a higher control rate of K loss (see before) provided the maximum rate of loss attainable was similar to that of estrogen-treated pieces.

In both estrogen-treated and estrogen plus progesterone treated pieces, dibenamine alone (present during the recovery period in Krebs–Ringer solution) decreased potassium loss significantly compared with that of untreated pieces. The addition of epinephrine or norepinephrine after dibenamine did not increase potassium loss compared with pieces without adrenergic blockade, but did increase potassium loss compared with pieces treated with dibenamine alone (1.6 and 0.8 meq/kg for epinephrine and norepinephrine in estrogen-pretreated pieces and 0.5 meq/kg for both drugs in pieces pretreated with progesterone as well). However, only the increased loss due to epinephrine in estrogen-treated pieces was statistically significant. Thus dibenamine appeared to have direct actions on potassium metabolism which may have added to the effects of adrenergic blockade. However, it should be noted that the expected change in tissue potassium concentration (increase relative to controls) in dibenamine-treated pieces was not obtained. No convincing explanation of this discrepancy could be found.

In all uterine pieces, irrespective of hormonal preparation, a secondary decrease in potassium output occurred following exposure to epinephrine during the second (60-minute) period. Despite this, the total loss of potassium into the medium over the entire interval in the potassium-deficient medium was greater in both types of drug-treated tissue, significantly so in the case of estrogen-treated pieces. Furthermore, the potassium concentration per kilogram dry weight of both types of tissue was diminished significantly. (Reference to dry rather than wet weight seemed preferable in view of the fact that progesterone had decreased the water concentration of some of these tissues.)

In Table III, the effect of 60 μg of epinephrine on the rate of potassium loss per kg wet weight per minute has been tabulated in selected experiments using procedure 3 (perfusion technique). Table IV summarizes similar data from pieces of pregnant cat uterus. Attention should be called to the fact that

TABLE III

Estrogen-treated rabbit uterus*—effects of epinephrine (60 μg) on rate of K loss

	K lost, kg^{-1} min^{-1}			
	Run 1	Run 2	Run 3	Run 4
Before epi.	0.58	0.71	1.13	1.08
During initiation of epi. contraction	1.48	1.35	2.09	Lost
During sust. contraction	0.81	0.46	1.20	1.80
	0.58	0.46	1.15	0.94
After partial relaxation	0.46	0.32	0.91	0.87
	0.44	0.32	0.91	0.87
Duration, min	12	14	17	18
Magnitude, g	3.5	5.5	4	4

*Runs 1 and 2 with a strip not spontaneously active. Runs 3 and 4 with a strip spontaneously active.

TABLE IV

Pregnant cats*

Effect of epinephrine (60 μg) on rate of K^+ loss in pregnant cat uterus

	K lost, kg^{-1} min^{-1}				
	Run 1	Run 2	Run 3	Run 4	Sham run
Before epi.	0.20	0.32	0.53	0.47	0.60
Onset epi. contraction	0.62	0.65	0.78	0.60	0.56
During sust. contraction	—	—	—	—	—
After relaxation	0.12	0.21	0.48	0.38	0.49
	0.12	0.21	0.48	0.38	0.50
Duration, min	2	2	5	4	—
Magnitude, g	5	12.5	5	11	0

*Runs 1 and 2 with a strip not spontaneously active. Runs 3 and 4 with a strip spontaneously active.

most of the extra potassium is found in the sample collected during the initial isometric tension increase and that during the following phase while the contraction was still sustained, the rate of potassium loss diminished toward (and in some cases beneath) the control levels. The duration of the epinephrine-induced contraction in the rabbit uterus was in every case greater than 10 minutes. This accounts for the finding that pieces studied by method 2 had shortened during the 10-minute exposure to epinephrine while their controls had lengthened. In runs 3 and 4, during which the tissues were contracting spontaneously prior to drug administration, the rate of K output per unit of tissue mass during the periods prior to drug administration was much higher than in similar periods of other runs. The additional effect of epinephrine was much less marked than in pieces which were initially quiescent.

The effect of relaxation of potassium output could not be distinguished because of the fact that the wearing-off of drug effects occurred gradually. There were intermittent periods of relaxation which became more complete and more prolonged as the effect of the drug wore off and there was never a

TABLE V

Responses of variously treated cat uterus to epinephrine and histamine

Type	Mechanical response (other pieces)	15-min intervals	Drug additions	K lost, meq/kg wet weight		
				Epi., 10 μg/ml	Control	Hist., 20 μg/ml
Anestrus	Inhibition	1	−	4.8	4.1	
		2	−	4.5	4.2	
		3	−	3.9	3.4	
		4	+	3.7	3.3	
		5	−	3.6	3.1	
		6	−	3.2	2.6	
		7	−	3.1	2.5	
				(137)*	(137)*	
Estrogen-treated	No effect	1	−	5.9	5.0	4.5
		2	−	6.1	4.7	4.9
		3	+	6.3	3.9	2.6
		4	−	4.3	4.2	3.3
		5	−	4.3	3.9	3.8
		6	−	3.7	3.8	3.9
		7	−	3.9	3.4	3.1
				(197)*	(195)*	(221)*
Post partum	Contraction	1	−	3.0	2.9	
		2	−	2.8	2.9	
		3	+	3.7	2.9	
		4	−	3.6	2.5	
		5	−	2.7	2.5	
		6	−	2.6	2.3	
		7	−	2.4	2.1	
				(115)*	(143)*	

*Tissue potassium content in meq/kg dry solids at end of experiment.

sudden and complete relaxation complementary to the abrupt contraction at the onset of drug action.

In Table V, selected data collected from cat uteri studied by procedure 1 (sampling procedure — delayed drug addition) have been summarized. These data indicate that the rate of potassium loss was increased and the residual potassium concentration decreased only in pieces which contracted in response to epinephrine; i.e. in the segment from the *post partum* uterus.[5]

Because of the failure of dibenamine to completely block its actions on potassium loss the question arose as to whether the effect of epinephrine on potassium loss was related to its glycogenolytic effects (see 4). Analyses were made of the glycogen content of the uterus after exposure to epinephrine, using a modification of procedure 2 (sampling procedure—immediate drug addition).

[5]In the uterine segment taken from a cat *post partum*, the rate of potassium loss was lower than in the other uterine segments. This admits the possibility that the increased potassium loss in this segment resulted from retention of more potassium and therefore of the ability to respond by increased ion loss rather than from an inherent difference in the tissues. This possibility was ruled out in other experiments in which the drug was added immediately on transfer to a potassium-free medium with similar results.

In the uterine segments from estrogen-treated cat, the potassium output was higher in the sample to which epinephrine was added than in subsequent samples. This probably represented carry-over of Krebs–Ringer solution since high values were also found in the previous two samples and since the final potassium concentration was as high in the tissue exposed to epinephrine as in the segments which was not.

TABLE VI

Effect of epinephrine in relation to glycolysis

	No.	K lost, meq/kg	% wt. change	Glycogen: Controls, g/kg orig. weight		Glycogen: Changes from controls, g/kg original wt.	
				Fresh	Krebs	Cf. fresh	Cf. Krebs
Estrogen							
Control	4	2.48	+0.5	5.0	5.0	−0.5†	−0.5†
Epinephrine*	4	4.76*	−7.2*†	—	—	−0.34	−0.37
Estrogen + progesterone							
Control	4	1.62	−1.8	3.2	2.8	−1.4†	−1.0†
Epinephrine*	4	4.06*	−8.4*†	—	—	−0.7†	−0.3

*Significant difference from control.
†Significant difference from zero.

The glycogen content of rabbit uteri was found to decrease very slightly after exposure to Krebs–Ringer solution (Table VI). On exposure to saline–bicarbonate without epinephrine a further loss of about 0.5 g/kg (estrogen-treated) and 1.0 g/kg (estrogen and progesterone treated) occurred. When epinephrine was added, there was a smaller loss of glycogen than in untreated pieces despite the increased K loss. Thus, epinephrine apparently does not produce an appreciable enhancement of glycogenolysis in uterine smooth muscle under these conditions.

Na and Water Exchanges

Epinephrine significantly diminished the large increase in sodium and water concentrations of rabbit uterine pieces which usually occurred in saline—bicarbonate (Table II). Dibenamine did not block this effect, at least not in estrogen-treated pieces. Too few chloride values were obtained to permit conclusions as to the effect of epinephrine in the uptake of this ion by uterine tissues.

Similar studies of cat uteri indicated that epinephrine treatment decreased sodium and water concentrations per kilogram dry weight (as compared with control segments) only in the four pieces (pregnant) in which it induced contraction (Na by an average of 71 meq/kg; range 52 to 140 meq/kg). In anestrus and estrogen-treated tissues, epinephrine was inhibitory or ineffective and did not alter sodium or water concentrations significantly.

Histamine, Acetylcholine, and Pitocin

In Table II, the effects of acetylcholine, histamine, and pitocin on cation exchange also are summarized. By the method used (sampling procedure 2 — immediate drug addition), none of these agents produced a demonstrable increase in potassium loss even though contraction probably had occurred. In fact, acetylcholine caused a significant decrease in net potassium outflow in both estrogen-treated and estrogen plus progesterone treated pieces. Histamine had an inhibitory effect on K loss only in pieces treated with progesterone. There were no significant effects on tissue uptake of sodium and water.

TABLE VII

Effect of histamine* (60 μg) and acetylcholine* (60 μg) on rate of K loss (kg^{-1} min^{-1}) in rabbit and cat uterus

	Run 1	Run 2	Run 3	Run 4
A. Histamine—estrogen-treated rabbit uterus				
Before hist.	0.46	0.44	1.15	1.26
Onset hist. contraction	0.91	1.85	1.84	1.84
During sust. contraction	0.71	0.70	1.08	0.79
After relax.	0.46	0.46	0.91	0.77
				0.75
Duration, min	5	5	3	3
Magnitude, g	3	2	2	3
B. Acetylcholine—estrogen-treated rabbit uterus				
Before ACh	1.35	0.46	1.20	1.15
Onset ACh contraction	1.68	2.00	1.61	1.20
During sust. contraction	1.25	0.71	1.15	1.20
After relax.	0.71	0.70	0.75	0.91
	0.67	0.43		
Duration, min	6	6	8	6
Magnitude, g	4	5	5	6
C. Histamine—pregnant cat uterus				
Before hist.	0.38	0.21	0.49	0.72
Onset hist. contraction	0.61	0.61	0.49	0.67
During sust. contraction	0.26	0.43	0.49	0.69
			0.78	0.72
After relax.	0.21	0.21	0.56†	0.74†
			0.53†	0.60†
Contraction duration, min	6	7	7	10
Contraction magnitude, g	7.5	12.5	7.5	11
D. Acetylcholine—pregnant cat uterus				
Before ACh	0.32	0.32	0.69	0.50
Onset ACh contraction	0.49	0.61	0.69	0.68
During sust. contraction	—	—	—	—
After relax.	0.35	0.27	0.28†	0.49†
	0.24	0.19	0.49†	
Contraction duration, min	2	2	6	4.5
Contraction magnitude, g	7.5	12.5	7.5	11

*Runs 1 and 2 with a strip not spontaneously active; runs 3 and 4 with a strip spontaneously active.
†Intermittent relaxation and contraction.

Initial results using the sampling procedures 1 as well as 2 (Table V) failed to provide evidence that any of these agents produced an appreciable increase in K loss, although all induce contractions in these tissues. However, the contractile effects of these agents are more fleeting than those of epinephrine. It seemed possible that a compensatory decrease in the rate of K loss during subsequent relaxation (10) might have obscured the anticipated effect. Therefore, procedure 3 was devised to test this hypothesis. These results (Table VII, A and B) showed clearly that acetylcholine and histamine, and presumably pitocin as well, caused an increased K loss during their contractile effect, unless the K loss had already been increased (Table VII A) in association with pre-existing spontaneous contractions.

In cat uterus, some studies of estrogen-pretreated uteri by the second method (sampling procedure — immediate drug addition) indicated that acetylcholine, histamine, and pitocin caused a decreased potassium loss, as in rabbit uterus. Moreover, in initial studies by the first method (sampling procedure — delayed drug addition) histamine had repeatedly failed to produce any increase in potassium outflow, and usually appeared to decrease it. However, in contrast to rabbit uterus these drugs increased the water and sodium concentration in cat uterine segments (an average increase of 94 meq/kg dry weight sodium and 10 g/kg wet weight in six experiments). The sodium uptake was much greater than could be accounted for by tissue inhibition of the bathing medium.

When pregnant cat uterus was studied by the third method (perfusion technique) the contractile effects of acetylcholine and histamine, and presumably pitocin, were usually accompanied by an increased potassium loss (Table VII, C and D). Thus it became apparent that previous failure had been due either to lack of contractile effects of these drugs under the conditions used or to the short durations of these effects relative to the 10-minute duration of exposure to the drug. When there was an increased initial rate of K loss as a result of spontaneous contraction, the effects of acetylcholine and histamine on K loss were minimal or absent (runs 3 and 4).

Effects of Electrical Stimulation on Electrolyte Exchange

Rabbit uteri were stimulated according to the method of Csapo and Corner (11). At the bottoms of Table II A and B, data have been summarized from parallel experiments using method 2 (sampling procedure — immediate drug addition) in which the effects of electrical and epinephrine stimulation have been compared. Electrical stimulation produced a more marked increase in the rate of potassium loss than did the drug, an effect which persisted even during the subsequent hour following stimulation. Electrical stimulation tended to increase residual tissue sodium concentrations, though it decreased both water and potassium concentrations. A relative increase in tissue sodium in the fact of a relative loss of tissue water suggested that the changes might be even more marked per kilogram wet weight. In fact with estrogen-treated pieces, electrical stimulation significantly increased the wet weight sodium concentration by 16.7 meq/kg while decreasing the potassium concentration only 8.5 meq/kg.

In the cat, similar electrical stimulation, though probably not optimal for these tissues[6] produced increased potassium loss from pieces from anestrus, estrus, estrogen-treated, estrogen and progesterone treated, and pregnant uteri. Representative values are summarized in Table VIII. The data did

[6]In the cat uterus, variation in the stimulating voltage did not affect the magnitude of the response as in the rabbit uterus. In the rabbit uterus, reduction of the voltage below about 10 v/cm caused reduced contraction amplitude. In the cat uterus, considerably smaller voltages resulted in a similar contraction. If the voltage was reduced to very low values, no contraction was recorded. These results are consistent with recent findings in this laboratory that action potentials are conducted over very short distances (<2 mm) in rabbit uterus and over considerably larger distances in cat uterus (>2 cm).

TABLE VIII

Effect of electrical stimulation of pieces of cat uterus for 10-minute interval on K loss

Condition piece	K loss (meq/kg wet wt.)		
	10 minutes	60 minutes	Total
Anestrus			
Stimulated	12.1	12.6	24.7
Control	7.5	12.3	19.8
Estrus			
Stimulated	5.6	8.3	13.9
Control	3.6	8.7	12.3
Estrogen			
Stimulated	13.2	10.1	23.3
Control	10.2	10.1	20.3
Estrogen+progesterone			
Stimulated	10.4	9.8	20.2
Control	6.1	10.7	16.8
Pregnant			
Stimulated	6.1	5.8	11.9
Control	4.6	5.6	10.2

not permit clear-cut evaluation of the effects of prior electrical stimulation on tissue sodium, water, and chloride, but suggested that all of these were decreased.

Effects of Calcium Ion and Long-term Effects of Epinephrine

Several results suggested that the epinephrine-induced effect on K loss might be different when the immediate contractile effects had disappeared. These included discrepancies as to its actions on K loss when studied at different intervals, the decreased loss into the medium in successive sampling intervals following exposure to epinephrine, and the failure of increased K loss into the perfusate to persist beyond the period of initial tension increase. In addition, earlier studies (13) indicated that after the excitatory contractile effects of epinephrine had worn off or had been blocked, inhibitory action on contractile function could be demonstrated. Preliminary studies made it apparent that the contractile action of epinephrine persisted for varying but sometimes prolonged intervals. Therefore a period of 120 minutes' duration was adopted and the lengths of the strips were measured before and after exposure to the drugs. In addition, the restoration of calcium to the saline–bicarbonate medium produced changes very similar to those induced by prolonged exposure to epinephrine. Furthermore, effects of the two substances were found to be additive in some instances.

In Table IX, typical results illustrating these effects have been summarized. The changes in solids and water were calculated assuming that the distribution of mass between these constituents was initially the same throughout the uterus, i.e. the same in the experimental and control pieces. Despite the lack of quantitative accuracy of this assumption in the rabbit (see Methods), the results clearly indicated that both epinephrine and calcium ion prevented or reversed the uptake of water occurring under these conditions in pieces of

TABLE IX

	Length, cm			Change in solids, g/kg fresh tissue	Change in H_2O, g/kg fresh tissue	K in solution, meq/kg orig. wt., 15–120 min	Milliequivalents/liter of electrolytes in tissue H_2O				H_2O, ml/kg	Vol. of distribution	
	Initial	Final	Δ				K	Na	Na + K	Cl		Na_{ap}	Cl_{ap}
Rabbit uterus 207—estrogen				149.6	850.4		83.2	83.3	166.5	62.1	850	488	480
RH-1 Epi.	1.4	1.5	+0.1	−14	+ 2	17.1	60.9	106.2	167.2	74.6	863	644	527
-2 Contr.	1.4	1.7	+0.3	−18	+78	16.5	44.2	142.3	186.5	83.5	876	789	537
-3 Epi. + Ca^{++}	1.4	1.6	+0.2	−17	−38	14.1	57.1	85.8	142.9	57.5	860	543	405
LH-1 Epi. + Ca^{++}	1.4	1.6	+0.2	−16	−39	13.3	62.3	93.3	155.6	59.2	858	589	416
-2 Contr.	1.3	1.5	+0.2	−11	+73	20.7	51.8	142.4	194.2	88.7	869	784	567
-3 Ca^{++}	1.5	1.5	0.0	−14	+50	12.7	58.6	113.7	172.3	83.0	868	667	532
Rabbit uterus 209—estrogen and progesterone				151.0	849.0		81.0	85.0	166.0	60.8	849	497	468
RH-1 Epi. + Ca^{++}	1.5	1.8	+0.3	−21	−70	11.6	80.6	92.0	172.6	65.6	852	577	458
-2 Contr.	2.0	2.1	+0.1	−18	+32	12.8	61.6	118.6	180.2	91.1	865	650	588
-3 Contr.	2.1	2.2	+0.1	−17	+22	11.3	57.9	117.3	175.2	86.4	867	645	551
LH-1 Epi.	1.8	2.1	+0.3	−23	−81	8.0	65.5	100.9	166.4	59.2	857	606	463
-2 Epi. + Ca^{++}	2.1	1.8	−0.3	−22	−59	7.2	60.8	88.4	149.2	62.5	859	558	440
-3 Ca^{++}	1.8	1.4	−0.4	−18	−52	15.1	59.9	107.7	167.6	78.6	857	624	534
Cat uterus 131—estrogen				188	812		85.5	105.3	190.8	89.0	812	553	617
RH-1 Epi.	1.5	1.5	0.0	−17	+57	14.7	47.3	103.2	150.5	105.3	838	558	664
-2 Contr.	1.4	1.3	−0.1	−17	+62	17.6	46.2	—	—	98.3	835	—	604
-3 Epi.+ Ca^{++}	1.6	1.6	0.0	−20	+69	9.2	52.5	100.8	153.3	93.7	837	570	591
LH-1 Epi.	1.5	1.5	0.0	−17	+57	5.2	51.8	95.8	147.6	81.5	835	540	512
-2 Contr.	1.5	1.6	+0.1	−19	+46	12.5	45.2	108.7	153.9	105.6	830	572	638
-3 Ca	1.7	1.7	0.0	−17	+59	7.6	53.1	104.9	158.0	87.8	835	580	541
Cat uterus 130—estrogen and progesterone				184	816		78.7	81.6	159.3	84.5	816	430	551
RH-1 Epi.	1.3	1.6	+0.3	−20	+17	14.1	56.5	98.0	154.5	128.8	833	527	806
-2 Contr.	1.3	1.6	+0.3	−16	+11	16.7	54.6	—	—	—	836	—	—
-3 Epi. + Ca^{++}	1.4	1.6	+0.2	−16	+25	14.0	60.4	93.0	153.4	82.9	830	526	522
LH-1 Epi. + Ca^{++}	1.3	1.3	0.0	−17	0	5.9	62.2	103.8	166.0	81.6	832	583	511
-2 Contr.	1.3	1.4	0.1	−12	+ 2	18.6	49.2	108.4	157.6	100.5	833	580	615
-3 Ca^{++}	1.6	1.6	0.0	−15	+17	12.6	52.8	124.2	177.0	93.0	833	685	572

rabbit uterus exposed to saline–bicarbonate alone. Epinephrine and calcium had little effect on water uptake in the cat. The loss of total solids was not markedly or consistently influenced in either species. As indicated by potassium loss (the early loss was not included in the values reported in order to decrease variation from carry-over of Krebs–Ringer solution) and by residual potassium concentrations, epinephrine and calcium both tended to reduce the amount of potassium lost over long intervals in both species.

In the rabbit uterus these substances also reduced the uptake of both sodium and chloride, particularly the latter. This is indicated by the volumes of distribution of these ions in the tissues. The combination of epinephrine and calcium had even more marked effects on all of these values. Their combined effects were particularly marked on chloride. Its volume of distribution remained constant or even decreased. In uterine segments from the estrogen-treated animals, the addition of both epinephrine and calcium led to a net reduction in the volume of distribution of sodium greater than the concomitant reduction in the volume of distribution of chloride. In pieces from progesterone-treated uteri, however, the volume of distribution of chloride was more markedly reduced than that of sodium by addition of calcium and epinephrine. This difference could be referred chiefly to a smaller uptake of sodium in progesterone-treated pieces exposed to saline–bicarbonate alone.

In pieces of cat uterus, which do not contract in response to epinephrine, neither this drug nor calcium had marked effects in preventing expansion of the volume of distribution of sodium in long-term experiments. In fact, calcium alone appeared to increase sodium space in some progesterone-treated pieces (see cat 130). Calcium had the usual effect on restricting the volume of distribution of chloride, but the volume of distribution of chloride was reduced much more by a combination of epinephrine and calcium. In contrast to the effects in rabbit uterus, epinephrine alone appeared to increase the volume of distribution of chloride.

Discussion

Effect of Progesterone on K Leakage and Glycogen Concentration

The data included in Table I and Fig. 1 suggest strongly that progesterone pretreatment increases the rate of leakage of potassium into potassium-poor solutions from rabbit uterine cells as compared with those cells from animals prepared by injection of estrogen alone. This increase does not appear to be attributable to an increased gradient of potassium between the cellular water and the saline–bicarbonate solution, since the calculated cellular potassium concentration was no greater in progesterone-treated uteri than in uteri treated with estrogen alone (see Table I and references 1, 3, and 14). This difference was not readily attributable to the various physical factors which limit the rate of diffusion since progesterone-treated segments were thicker and had a longer average diffusion path and a smaller ratio of surface area to volume than segments pretreated with estrogen alone. Thus, the actual effect of progesterone on the potassium metabolism of rabbit uterine cells

probably is even greater than the analytical data revealed. Therefore, progesterone may act to increase the permeability of the cell membrane to potassium or to alter the rate of release of bound potassium (3).

Previously, evidence (1) has been presented that progesterone decreases the permeability of uterine cells to chloride during exposure to various artificial media. The present finding is of interest in view of the hypothesis (11, 15) that progesterone produces a negative "staircase" in rabbit uterus as a result of an alteration in cellular potassium balance such that repeated stimulation at short intervals causes a progressive depletion of the cellular potassium concentration below that necessary for optimal contractions. The results presented here show that in the resting state, the tendency to leak potassium was greater in the progesterone-treated rabbit uterine cells than in those treated with estrogen alone. Stimulation of contraction (both pharmacological and electrical) tended to increase the rate of potassium loss from rabbit uterine cells (relative to the control rate), but the increase was less marked in progesterone-treated pieces than in those treated with estrogen alone. Whether the difference reported here could account for the negative "staircase" of rabbit uterus cannot be decided until the effects of variation in intracellular potassium concentration on tension are known in uteri under various endocrine influences.

The progesterone-treated uteri had a lower glycogen content than those treated with estrogen alone, and tended to utilize their glycogen more rapidly in Krebs and in K-free solutions. Whether these differences were related to the difference in potassium leakage cannot be determined from presently available data. Caution in the interpretation of this correlation also is demanded by the fact that others (16) have found somewhat smaller concentrations of glycogen in estrogen-treated rabbit uteri and no difference between these values and others for uteri treated with progesterone as well. The finding that epinephrine tended to promote glycogenesis (or at least opposed glycogenolysis) is of interest in view of a similar action reported for the rat uterus (16), a tissue which usually relaxes in response to epinephrine.

Effects of Excitant Stimuli on Ion Exchange

The most important conclusion from this work has to do with the relation of contraction to potassium loss. All the drugs studied were shown to increase the loss of potassium when they initiate a contraction and this phase of their action could be experimentally isolated. Thus epinephrine, norepinephrine, histamine, and acetylcholine caused increased potassium loss from the uterus during the period of increasing tension at the onset of their contractile action. The action of epinephrine on potassium loss was largely blocked by dibenamine. It was absent in uteri which were not stimulated by this compound (e.g. uterus of estrogen-treated cat).

It should be emphasized that these data provide no basis for a conclusive decision as to whether these events (contraction and increased potassium loss) are causally related and if so, whether the contraction causes the potassium loss or vice versa.

Contraction did not appear to be associated with an increased sodium uptake. A definite conclusion with respect to sodium uptake during the rising phase of contractile tension cannot be made because sodium concentration was not studied in uteri analyzed at the peak of the initial contractile action (before recovery processes could have occurred) and because no satisfactory method of determining sodium concentration in cells is available (2). However, the diminished water uptake resulting from exposure to epinephrine and norepinephrine was insufficient to account for the diminished sodium uptake, even if all the water affected by these drugs was assumed to be located in the extracellular space. Thus epinephrine and norepinephrine probably diminished uptake of sodium into cells while they increased potassium loss. Such a finding would tend to exclude a reciprocal exchange of sodium for potassium across smooth muscle cell membranes.

Electrical stimulation produced an increased uptake of sodium as well as increased rate of potassium loss in short-term experiments. The tissue chloride values were highly variable and the source of their variations not determined (2) so that precise localization of the site of sodium uptake is not possible. Spontaneous action potentials and contractile function in uterine smooth muscle are nearly independent of external sodium concentration and therefore of an inward sodium current (3). The failure to obtain increases in cell sodium concentration during stimulation would be in harmony with this finding.

Origin of the Potassium Lost during Stimulation

In experiments of this type, changes in rates of electrolyte exchange commonly are attributed to changes in the rate of ionic flux across the cell membranes. An alternate possibility is that electrolyte changes occurring during the contractile action of drugs (increased loss of potassium, decreased uptake of sodium, chloride, and water) were simply the result of a temporary concomitant decrease in extracellular volume. Several facts were consistent with this hypothesis. (*a*) The direction of the electrolyte changes observed (Na, K, and H_2O concentrations diminished). (*b*) The fact that increased potassium loss occurred chiefly during the phase of increasing tension rather than throughout the contraction. (*c*) The fact that any change which prevented an active increase in tension (selective blockade, use of unresponsive type of uterus) prevented the usual electrolyte responses to drugs. (*d*) The failure of histamine, acetylcholine, and pitocin to cause noticeable alterations in electrolyte concentrations in experiments of 10 minutes or more duration, inasmuch as contractions in response to these drugs did not persist for as long as the interval of exposure. (*e*) The finding that the effect of epinephrine on electrolyte exchanges varied with the duration of exposure and could be related to whether the shortening effect of the drug still persisted.

However, quantitative considerations make acceptance of this explanation difficult. Epinephrine and norepinephrine increased potassium output 2.6 and 2.0 meq/kg wet weight, respectively, in uterine pieces from estrogen-pretreated rabbits over a 10-minute period. In uterine pieces which were

equilibrated with Krebs solution prior to placing in K-free saline, the extracellular fluid contained 5.75 meq/liter of potassium. The extracellular fluid volume (ECFV) was no more than 400–500 ml/kg so that extracellular potassium was less than 2.2 to 2.87 meq/kg of tissue. Thus abolition of all or nearly all of the extracellular volume would be required to account for the increased K loss on this basis alone (in the endometrium as well as in the myometrium). Thus while diminution of the extracellular space during contraction might contribute to the observed ionic effects, this mechanism appears to be inadequate to account for the magnitude of these changes.

Drug-induced effects in the continuous perfusion experiments also conform with this conclusion. The output of potassium in the unstimulated state decreased rapidly to an approximately stable value of 0.3 to 1.0 meq/l. minute after about 5–6 minutes. Thus, at the initial concentration of potassium in the ECFV (5.75 meq/l.) K loss from this tissue ECFV was initially greater than replacement from cells. Stability of the rate of loss from tissues probably was attained when the extracellular potassium concentration had decreased to the point at which loss to the medium was balanced by gain from the cells. The rate of tissue K loss was 0.75 to 2.5 meq/liter cell (or extracellular) water per minute, taking the values: tissue water = 80%; cellular water = 50% of tissue water (see 1). The net potassium loss per minute was large in comparison with the total extracellular potassium at any moment, since this must have been less than 5.75 meq/liter. In samples collected during contraction, 0.2 to 1.5 meq/kg wet weight tissue/minute was added to the basal rate of K loss. This, likewise, would represent a large fraction (up to > 26%) of the extracellular potassium. If this change occurred only in the muscular portions of the tissue, a reduction of ECFV of 52% or more would be required if this were the sole mechanism of the changes. Again, the quantitative requirements of this hypothesis make it difficult to accept.

Mechanism of Potassium Loss during Contraction

The above considerations suggest strongly that the major part of the potassium added to the basal outflow into a K-free medium during the rising phase of the induced contraction was cellular in origin. There is at present no secure basis for determining the mechanisms responsible for this effect. A selective and continuous increase in potassium permeability would probably result in corresponding hyperpolarization of the membrane, a change which has never been observed during spontaneous contractions (3). Depolarization during the action potentials of uterine muscle would decrease the electrochemical gradient to potassium and permit increased outflow. Repolarization of the membrane during each action potential could result from loss of potassium, either as a result of this decrease in electrical potential alone, or aided by a concomitant increase in membrane permeability to K.

In striated muscle cells, sodium usually enters cells in exchange for the potassium lost during stimulation. No apparent increase in cellular sodium concentration was observed during drug stimulation in these studies. In fact,

the total tissue sodium decreased. Electrical stimulation did cause an increase in tissue sodium concentration and therefore may involve mechanisms affecting membrane permeability different from those accompanying drug stimulation. However, the evidence available can not exclude the possibility that cellular sodium uptake accompanies drug-induced contractions during the rising phase of tension.

Results already published (2) have provided evidence that active accumulation of potassium occurs in smooth muscle tissues. Furthermore, uterine action potentials accompanying spontaneous contraction are independent of sodium concentration in the external medium over a wide concentration range (3). The possibility that drugs also may act on potassium exchanges of smooth muscle independent of effects in sodium exchange appears plausible. Other mechanisms involving alterations in the flow of cellular ions passing through the cell membrane of smooth muscle also have been considered (3).

In addition, the failure of increased potassium outflow to persist throughout the increase in tension must be explained in terms of the changes in electrical properties of the membrane. Perhaps action potentials do not persist beyond the rising phase of the contraction during drug-induced contractions. Further study of membrane potentials and concomitant ion exchange during drug-induced contractions may clarify this problem.

The changes reported here resemble those obtained with taenia coli (10) in which increased quantities of radioactive potassium were released from labelled tissue potassium during periods of increasing tension. The occurrence of a complementary decrease in potassium outflow during relaxation was reported in taenia coli. The existence of decreased potassium loss during relaxation of uterine muscle can be inferred from the different results obtained with histamine and acetylcholine when studied by methods No. 1 and 2 versus No. 3. Here, the output of potassium during the phase of increasing tension was shown to be elevated so that there must have been a secondary decrease in potassium output, presumably during relaxation, to account for the negative findings using methods 2 and 3. (These procedures used long sample intervals which probably included relaxation as well as contraction.) The decreased loss of potassium on relaxation of uterine muscle was not detectable directly because relaxation was never abrupt, but always was partial and intermittent.

The mechanism whereby epinephrine was able to produce an increased potassium loss under experimental conditions (methods 1 and 2) which obscured the similar effect of other contractile agents (histamine, acetylcholine, and pitocin) requires explanation. Epinephrine in the concentrations used produced a prolonged contractile effect so that a complementary decrease in potassium loss did not occur during the same sampling interval (10–15 minutes). If tissues were exposed to epinephrine for longer intervals (120 minutes) so that the shortening effect of this drug had disappeared, epinephrine also decreased potassium loss. This fact rules out the possibility that epinephrine might increase potassium loss during or after relaxation in contrast to the other

contractile agents. So far then, there is no reason to suggest unique actions of epinephrine on potassium metabolism of uterine muscle, although this possibility has not been excluded entirely.

The Effect of Prolonged Exposure to Epinephrine and Calcium Ions on Uterine Electrolyte Exchanges

Prolonged exposure to epinephrine had actions on rabbit uterine electrolyte exchange which require comment. As with shorter durations of exposure to the drug, the uptake of water and sodium which occurs in the saline–bicarbonate medium was diminished. The restoration of calcium ions to the medium had a similar though less marked effect. In the presence of calcium ions, epinephrine still exerted an additional effect on water and sodium uptake. In the case of water, the combined effect of calcium and epinephrine caused a net loss compared with the initial water content. These relationships were particularly clear in the estrogen-treated uterine pieces but in those treated with additional progesterone were obscured by variation related to position of the pieces within the horn. In those estrogen-treated pieces which lost water during prolonged exposure to epinephrine, there was also a diminution in the volume of distribution of chloride compared both with its original volume of distribution and with the increased volume of distribution in pieces exposed to the saline–bicarbonate in the absence of drug. This suggests that the water lost may have been, at least partly, extracellular in origin. Despite this presumed diminution of extracellular space, the sodium volume of distribution increased in such pieces, so that, presumably, sodium still was penetrating into cellular water, though to a lesser extent than in those pieces exposed neither to calcium nor to epinephrine.

In those uterine pieces, potassium loss was diminished by addition of epinephrine and calcium to the saline–bicarbonate medium. Thus, in this instance a diminished uptake of sodium into cells may have been linked with a diminished loss of potassium from cells. However, the interpretation was not applicable to the effects of shorter period of exposure to saline–bicarbonate (see Table I) or to the short term effects of epinephrine on these exchanges (see Table II and previous discussion).

In connection with the question of whether or not reciprocal exchange of sodium for potassium occurred in cat uterine pieces exposed to saline–bicarbonate for long intervals, it is of interest that calcium ion or calcium ion and epinephrine diminished the loss of potassium into saline–bicarbonate but did not decrease the penetration of sodium into tissue water. Calcium did prevent further penetration of chloride into tissue water. If this restriction of chloride penetration was equivalent to restriction of expansion of the extracellular space, then sodium must have penetrated cellular water to a greater degree in the presence of calcium ion, despite the diminished loss of cellular potassium. Obviously, the occurrence of tightly linked reciprocal movements of sodium and potassium is questionable in uterine tissues. This question is now being explored more fully.

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NOTES

A PAPER CHROMATOGRAPHIC METHOD FOR THE PURIFICATION OF SHIKIMIC ACID-U-C^{14} OBTAINED FROM CULTURE FILTRATES OF A MUTANT OF ESCHERICHIA COLI[1]

HAROLD J. PERKINS[2] AND S. ARONOFF

During the course of a study of the chemistry of boron deficiency in higher plants (1), shikimic acid uniformly labelled with C^{14} was required. Since this material was not commercially available and since the method of Srinivasan *et al.* (2) was not practical for the preparation of milligram amounts or less, a paper chromatographic procedure was developed for the purification of shikimic acid-U-C^{14}. The details of this procedure are presented in this note.

Uniformly C^{14}-labelled shikimic acid was obtained from culture filtrates of Davis' 83-24 mutant of *Escherichia coli*, strain W (2, 3, 4) grown on glucose-U-C^{14} as the sole source of carbon. The composition of the nutrient solution in which the organism was grown was that recommended by Srinivasan *et al.* (2), modified by the addition of $MnSO_4.H_2O$, $FeSO_4.7H_2O$, and $CaCl_2.2H_2O$ (1×10^{-4}, 5×10^{-4}, and 0.01 g/l., respectively).

For preparation of the shikimic acid-U-C^{14}, 0.1 ml of a heavy suspension of the *E. coli* mutant was added to 10 ml of the nutrient solution containing 50 mg of glucose-U-C^{14} (prepared from ethanolic extracts of soybean leaves after 1 hour of photosynthesis in $C^{14}O_2$) in a 40-ml, conical centrifuge tube. The mixture was incubated at 35° C with continuous, vigorous shaking on a wrist-action shaker. After 48–56 hours of growth, the culture was acidified to pH 6.0 by the dropwise addition of 1 *N* HCl, heated to boiling to coagulate the protein, and the bacterial cells were removed by centrifugation. The pellet was washed thoroughly with distilled water and the combined supernatants and washings were concentrated, under reduced pressure, to a small volume. The yellow solution so obtained was banded onto sheets of Whatman No. 3MM filter paper and chromatographed ascendingly in *n*-butanol – acetic acid – water (100:6:25). This solvent afforded clean separation of the shikimic acid ($R_f = 0.40$) from 5-phosphoshikimic acid ($R_f = 0.05$), both of which accumulated in the culture medium of this mutant. The band of shikimic acid-U-C^{14} was located by spraying the edges of the chromatograms with 0.01 *M* aqueous potassium periodate solution followed, after a five-minute drying period at room temperature, by overspraying with a solution the composition of which was as follows: 35% saturated sodium tetraborate, 0.8% potassium iodide, 0.9% boric acid, and 3% soluble starch (5). The

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latter solution was boiled to dissolve the starch. The shikimic acid appeared, after a few minutes at room temperature, as a non-permanent white spot on a blue background.

After elution of the radioactive shikimic acid from the paper with distilled water, the aqueous solution was extracted twice with peroxide-free diethyl ether, concentrated under reduced pressure to a small volume, and rebanded onto Whatman No. 3MM filter paper. The descending chromatogram was developed with phenol – water – 98% formic acid (3:1:0.04) in which the R_f of shikimic acid was 0.30.

The radioactive shikimic acid obtained using this procedure was inseparable chromatographically from authentic shikimic acid in benzyl alcohol – isopropanol – *tert*-butanol – water – 98% formic acid (3:1:1:1:0.12), *n*-propanol – conc. ammonium hydroxide (70:30), and *n*-butanol – 98% formic acid – water (100:5:20). Autoradiography of such chromatograms showed no radioactive contaminants to be present in the prepared compound. The yield of shikimic acid-U-C^{14} was four to five per cent based on the total radioactivity present initially as glucose-U-C^{14}.

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